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IMMUNOLOGICAL APPROACH TO THE IDENTIFICATION AND DEVELOPMENT OF VACCINES TO VARIOUS TOXINS

FINAL REPORT

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Monoclonal antibodies of relatively high binding affinity constants specific for the sodium channel blockers saxitoxin and tetrodotoxin, and the synthesis inhibitor mycotoxin T-2 were generated. Two monoclonal antibodies against saxitoxin and two against tetrodotoxin were isolated and shown to inhibit the binding of saxitoxin and tetrodotoxin, respectively, to rat brain cell membrane. They were also effective in protecting against the saxitoxin and tetrodotoxin-induced reduction of peripheral nerve action potentials in rat tibial nerve when administered in situ. Polyclonal rabbit, and monoclonal anti-idiotypic antibodies have been generated and characterized. An IgG_{1k} mAb specific for T-2 was also generated. This antibody, termed HD11 (IgG_1), completely protected the human cell lines Hep-2 and KB against T-2-induced cytotoxicity. A monoclonal anti-idiotypic antibody (DE8) specific for HD11 anti-T-2 was generated with the following characteristics: 1) DE8 bound to HD11, but not to other mouse IgG_1 antibodies or to normal mouse immunoglobulins; 2) DE8/HD11 interactions can be

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19. inhibited by free T-2; 3) DE8 completely abolish the protective effect of HD11 against T-2 cytotoxicity; 4) injection of DE8 into syngeneic mice induced an HD11-idiotype-positive anti-T-2 antibody response that is protective against the in-vitro and in-vivo toxicity of T-2. This represents the first demonstration of the successful use of an antibody-(anti-idiotype) based vaccine against a nonproteinaceous, small molecular weight biological toxin.

FOREWORD

In conducting the research described in the report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources - National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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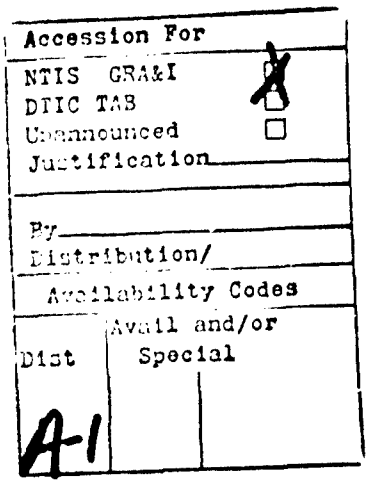

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I. INTRODUCTION

Because of the chemical nature and structure of the sodium channel blockers saxitoxin (STX) and tetrodotoxin (TDT), and the mycotoxin T-2, and their potent toxicity, conventional approaches in developing vaccines against these toxins such as synthetic peptide and recombinant technologies, or immunization with attenuated forms of the native antigen may not be practical. Indeed, conjugation of toxins such as T-2 to protein carriers has been shown to result in unstable complexes with potential release of the free toxin in its active form (1). Thus, it appears that a reasonable approach to vaccine development against potent biological toxins such as T-2 may be the anti-idiotype (anti-Id) based approach since the very toxic nature of the antigens preclude their use as immunogens.

Immunoglobulin (Ig) Id are defined as antigenic determinants associated with the variable region of the Ig molecule (2). Id can be associated with the antigen binding site or with the framework (nonbinding) site within the Ig variable region. Anti-Id (or Ab2) are anti-Ig antibodies that can be used to serologically define Id (or Ab1). Jerne's original network hypothesis of Id-anti-Id immune regulation suggest that some anti-Id antibodies may mimic the three-dimensional conformation of the nominal antigen (Ag) and thus serologically "behave" as the Ag (3). Such anti-Id antibodies are said to carry the "internal image" of the Ag and have been referred to as $Ab2_\beta$ (4,5). Because of their conformational mimicry of the nominal Ag, it is theoretically feasible to substitute $Ab2_\beta$ for the nominal Ag in the induction of a specific immune response. Indeed, a large number of studies have suggested the potential use of anti-Id antibody-based vaccines in a variety of infectious diseases and in controlling some tumors (4-13). Interestingly, administration of anti-Id against monoclonal anti-progesterone antibody has recently been shown to block pregnancy in mice (14).

We have generated a series of murine monoclonal antibodies (mAbs) specific for STX, TDT and T-2, respectively. Monoclonal antibodies which provide protection against toxicity induced by these biological toxins were used in syngeneic system to induce monoclonal and polyclonal anti-Id antibodies. The potential effectiveness of the anti-Id antibodies to serologically mimic the nominal antigen and to induce a protective immune response against toxicity was assessed.

II. RESULTS

The main objectives of this research work are to develop specific murine monoclonal antibodies of high binding affinities against small molecular weight toxins such as the trichothecene mycotoxin T-2 and the sodium channel blockers tetrodotoxin and saxitoxin, and to assess the potential development of anti-Id based vaccines in order to induce a systemic and protective immunity against these toxins. The following represents a summary of the achievements accomplished.

A. The Trichothecene Mycotoxin T-2 System.

A general problem that has been encountered throughout this research is the difficulty in generating monoclonal antibodies with relatively high binding affinity against compounds with small molecular weights. It is believed that this difficulty results from the nature of the small size of these compounds. As a result, various manipulations have been implemented in order to attempt to enhance the likelihood that monoclonal antibodies of high affinity can be generated. These will be discussed in a later section of the proposal.

After several attempts at fusing spleen cells from mice immunized with T-2 conjugated to large protein carriers such as ovalbumin (OVA) and bovine serum albumin (BSA) with the mouse myeloma NS-1 cell line to obtain mAbs specific for T-2, we were successful in isolating a mAb specific for T-2, designated HD11, which is protective against T-2-induced cytotoxicity (15).

1. Specificity of HD11 Monoclonal Anti-T-2 Antibody.

Figure 1 shows the binding of purified HD11 mAb to microtiter wells coated with 50 ul of 50 ug/ml of T-2-BSA in a binding enzyme-linked immunosorbent assay (ELISA). HD11, an IgG_1 mAb, specifically bound to T-2-BSA in a dose-dependent fashion, whereas no significant binding was observed with a control mAb of the same isotype.

To further assess the binding specificity of HD11, an inhibition ELISA was employed (Fig. 2). Free T-2 specifically inhibited the binding of HD11 to T-2-BSA-coated microtiter wells, whereas dilutions of free saxitoxin (SXT) or tetrodotoxin (TDT) did not result in significant inhibition of binding. Approximately 28% inhibition of the binding was observed with as little as 6.0 ng/ml of free T-2 toxin. On the other hand, as much as 400 ng/ml of SXT or TDT inhibited the binding of HD11 to T-2-BSA by less than 10%. The relative binding affinity constant of HD11 was determined to be approximately 4.8×10^{-8} by IC_{50} logit analysis (15).

2. HD11 Protects Against In-Vitro T-2 Cytotoxicity.

In order to determine whether HD11 can protect against T-2 induced cytotoxicity, we first titrated the T-2 toxin for its cytotoxicity on the human epidermoid cell lines Hep-2 and KB. Different amounts of T-2 toxin were added to monolayers of Hep-2 or KB cells in microtiter culture wells followed by the addition of [^{3}H]-leucine. Uptake of labeled leucine was then determined by scintillation counting. The cytotoxicity titration curves are shown in Figure 3. It is estimated from the curves that the concentrations of

T-2 required to inhibit 50% of the [^{3}H]-leucine uptake (IC_{50}) were approximately 10.0 ng/ml and 16.0 ng/ml of free T-2 for the KB and Hep-2 cell lines, respectively. When T-2 concentrations giving 50% inhibition of leucine uptake were incubated for 1 hr. with HD11 before addition to the KB or Hep-2 monolayers, the cytotoxic effect of T-2 was completely abolished (Table 1). With the Hep-2 cell line, 16.0 ng/ml of T-2 resulted in a leucine uptake that was approximately 62% less as compared to that of Hep-2 cells cultured in the absence of T-2. Addition of as little as 1.0 ug/ml of HD11 almost completely abolished the cytotoxicity of T-2 (3.6% inhibition of leucine uptake). Similarly, addition of T-2 preincubated with HD11 to monolayers of KB cells significantly enhanced the uptake of labeled leucine as compared with that of T-2 treated cells. The protective effect of HD11 on T-2 toxicity was not as pronounced as that observed with Hep-2 cell line. Incorporation of a control mAb of the same isotype but different specificity had no significant effect on the cytotoxicity of T-2 toxin.

The protective effect of HD11 on the in-vitro cytotoxicity of T-2 led to our attempt to investigate its potential passive in-vivo protection. Groups of BALB/C mice were injected i.p. with either saline, 2.0 mg/mouse purified HD11 or purified control mAb 2 hrs before i.p. administration of various doses of T-2. The results are presented in Table 2. The LD_{50} of T-2 in mice treated with saline and control mAb were 14.3 mg/kg and 13.7 mg/kg, respectively. The LD_{50} in mice pretreated with HD11 was 17.3 mg/kg. Although there was a slight increase in the LD_{50} of T-2 in mice treated with HD11 as compared to the control groups, the increase was not significant.

Although HD11 anti-T-2 mAb did not appear to provide mice with passive protection against T-2 toxicity, we proceeded to generate both rabbit polyclonal and murine monoclonal anti-idiotypic antibodies based on the reasoning that active immunization with anti-idiotype, which should generate a T-2-specific immune response, may be more effective in providing protection than passive immunization and that HD11 was quite efficient in providing protection against the in-vitro cytotoxicity induced by T-2.

3. Generation and Characterization of Anti-Idiotypic Antibodies Against HD11.

a) Polyclonal Rabbit Anti-Idiotype. To generate rabbit anti-idiotypic antibodies, HD11 purified by adsorption to and elution from protein A-agarose column and injected into two New Zealand white rabbits. The immunizations were given intramuscularly as an alum precipitate. After the fourth injection, the rabbits were bled and their sera were adsorbed repeatedly on a normal mouse Ig-Sepharose 4B to remove all anti-mouse Ig reactivity. Following five adsorptions of the rabbit sera with normal mouse Ig, the anti-isotype and anti-allotype reactivity was completely removed. The adsorbed rabbit sera was then tested for its specificity for HD11 employing HD11-coated microtiter wells (Fig. 4). The rabbit anti-Id reacts with HD11 in a dose-dependent fashion. The 50% binding end-point was determined to be approximately at a dilution of 1:320 of the rabbit anti-Id. The control rabbit anti-Id derived from an antibody specific for hepatitis B surface antigen did not react significantly with HD11 at a dilution of 1:20 (15). The rabbit anti-HD11 did not react with normal mouse Ig nor with mAbs of irrelevant specificities (data not shown).

To determine whether the rabbit anti-HD11 recognized the antigen-binding site of HD11, various concentrations of the rabbit anti-HD11 were preincubated for 1 hr with a constant amount of HD11 before addition to T-2-BSA-coated microtiter wells. The results shown in Figure 5 suggested that the rabbit anti-Id serum was efficient in inhibiting the binding of HD11 to T-2. Approximately 32 ug/ml of the rabbit anti-Id inhibited the binding of HD11 to T-2 by 50%, whereas the control rabbit anti-Id (rabbit antiserum against an anti-hepatitis antibody) did not show significant inhibition even at a concentration of 512 ug/ml. However, T-2 at concentration as high as 200 ug/ml added to HD_{11}-coated microtiter wells was unable to inhibit the rabbit anti-Id from binding to HD_{11} (data not shown). Taken together, these results suggest that the rabbit anti-HD11 anti-Id serum recognizes at least two distinct Id determinants, one which is associated with the binding region of HD11 and one which is associated with the framework or non antigen-inhibitable region. This finding is not surprising considering the polyclonal nature of the rabbit anti-Id serum.

b) Generation and Characterization of Mouse Monoclonal Anti-Idiotypic Antibody. To generate monoclonal anti-Id antibodies against HD_{11}, HD_{11} mAb was conjugated to KLH and alum precipitated. After four injections, the mice were sacrificed and their spleen cells fused with the NS-1 myeloma cell line. Supernatants obtained from wells with hybrid growth were screened using the following ELISAs. Microtiter wells were precoated with 50 microliters of ten micrograms per ml purified solution of HD_{11}. Nonspecific sites were blocked with PBS supplemented with 5% normal goat serum. Culture supernatants were then added to the wells and incubated for 1 hr at 37°C. After washing away unbound antibodies, reactivity was developed with HD_{11} conjugated to biotin (HD_{11}-b) and addition of the appropriate substrate (16). This assay is designed to assess the ability of the culture supernatants to bind to HD_{11}. A second ELISA was also performed concurrently. In this assay, microtiter wells were coated with 50 ul of a 50 micrograms per ml of T-2-BSA. Mixtures of a concentration of HD_{11}-b which gave approximately 50% T-2 binding and culture supernatants were added to the wells. Reactivity was developed by addition of avidin-horseradish peroxidase (A-HRP) and the appropriate substrate. This ELISA is designed to test for the ability of hybrid supernatants to inhibit the binding of HD_{11} to T-2. Among approximately 800 hybrids tested, one was found that bound HD_{11} and inhibited the binding of HD_{11} to T-2-BSA- coated wells. This hybrid was subcloned by limiting dilution until >95% of the clones were reactive. One subclone, designated DE8, was chosen for further characterization.

DE8 Isotype.

The isotype of the monoclonal antibody produced by DE8 hybrid cells was determined using two methods. The first method involves the direct staining of DE8 hybrid cells with goat anti-mouse isotype specific antibodies conjugated to FITC. The second method uses an isotyping ELISA kit (Biorad) as specified by the manufacturer. Data obtained from both methods indicated that DE8 is an IgG_{2b} monoclonal antibody.

Specificity of the Binding of DE8 to HD_{11}:

DE8 hybrid cells were injected into primed BALB/c mice to generate ascites. DE8 monoclonal IgG_{2b} antibody was purified from the ascites by caprylic acid precipitation. To assess the specificity of the binding of DE8

to HD_{11}, binding and inhibition ELISAs were employed (Figure 6 and 7, respectively). In the binding assay, various concentrations of purified DE8 antibody were added to HD_{11} precoated wells. Binding activity was detected by the addition of HD_{11}-b and the appropriate substrate. As shown in Figure 6, the binding of DE8 to HD_{11} was dose-dependent. The 50% binding end point was obtained at approximately 7.8 nanogram per ml of DE8. Normal mouse serum and a control monoclonal antibody (S3E.2) did not show any significant binding to HD_{11} (data not shown). To further assess the specificity of binding, an inhibition assay was used. In this assay, various concentrations of DE8 were mixed with a constant concentration of HD_{11}-b which gave 50% binding and were added to wells pre-coated with T-2-BSA. For negative control, normal mouse serum and control monoclonal antibody were used instead of DE8. Figure 7 shows that DE8 was very efficient in inhibiting the binding HD_{11}-b to T-2-BSA. As little as 3.6 ng/ml of DE8 inhibited the binding of HD_{11}-b binding to T-2-BSA by approximately 45%. On the other hand, normal mouse serum or control antibody did not inhibit the binding (data not shown).

DE8 Recognizes A Hapten Inhibitable Idiotypic Determinant:

To determine whether the idiotypic determinant recognized by DE8 is hapten-(T-2) inhibitable, the following two assays were done. In the first assay, various concentrations of free T-2 were used to inhibit the binding of HD_{11}-b to DE8-coated wells. The results are shown in Figure 8. T-2 specifically inhibited the binding in a dose-dependent fashion. The 50% inhibition end point was obtained with approximately 10 nanogram per ml of free T-2. Saxitoxin and tetrodotoxin at concentrations as high as 200 micrograms per ml did not show any significant inhibition. In the second ELISA, various concentrations of free T-2 were used to inhibit the binding of DE8-b to HD_{11}-coated wells. The results are presented in Figure 9. Similar to data obtained in Figure 8, T-2 was efficient in inhibiting the binding of DE8-b to HD_{11}. Again, there was no significant inhibition with saxitoxin or tetrodotoxin at concentrations as high as 200 micrograms per ml. Taken together, these results suggest that DE8 recognizes an idiotypic determinant which is specific for HD_{11} and which can be inhibited by free hapten T-2.

DE8 mAb Abrogates the Protective Effect of HD11 Against T-2 Cytotoxicity.

Since DE8 mAb appeared to recognize an Id determinant associated with the T-2 binding site on HD11 mAb, we tested the ability of DE8 to inhibit the protective effect of HD11 on T-2 cytotoxicity. Various concentrations of DE8 mAb were incubated with a protective concentration (100 ug/ml) of HD11 followed by the addition of a concentration of T-2 (16.0 ng/ml) predetermined to inhibit approximately 50% of the uptake of [^{3}H]-leucine by Hep-2 cells. The results are shown in Figure 10. HD11 at 100 ug/ml effectively protected against T-2 cytotoxicity, as previously shown. The [^{3}H]-leucine uptake in cultures with T-2 was 58, 156 + 3,214 cpm, whereas that in cultures containing T-2 and HD11 was 138,747 + 9,341 cpm which is comparable to the uptake of the Hep-2 cells cultures in the absence of T-2 (140,660 + 11,019 cpm). DE8 concentrations of 50 ug/ml and above completely negated the protective effect of HD11, as indicated by a comparable uptake of [^{3}H]-leucine between cultures without HD11 (58,156 + 3,214 cpm). As the concentrations of DE8 mAb decreased, there was a parallel increase in the uptake of radioactive leucine. The protective effect of HD11 was fully restored with DE8 concentrations of 12.5 ug/ml or lower. The control anti-Id has little if any effect on T-2 cytotoxicity at a concentration of 200 ug/ml.

DE8 Monoclonal Antibody is not Cytotoxic for Hep-2 Cells:

Since DE8 monoclonal anti-idiotypic antibody appears to carry the internal image of T-2, we tested the possibility that DE8 itself would mimic T-2 and thus would be cytotoxic to the human cell line Hep-2. The results indicated that DE8 itself was not cytotoxic to the Hep-2 cell line (data not shown). The lack of cytotoxicity may be due to the relatively large size of the DE8 antibody molecule as compared to T-2.

Inhibition of Binding by T-2 Metabolites:

The fine specificity of HD_{11} binding to T-2 was assessed in inhibition assays using T-2 metabolites which are structurally similar to T-2 (Appendix I). Table 3 shows the results of the inhibition of HD_{11} binding to T-2 BSA by 11 different T-2 metabolites. The results indicated that removal of the bulky alkyl side chain profoundly affected the binding of HD_{11} to T-2, suggesting that the fine specificity of HD_{11} is directed toward the alkyl side chain of the T-2 molecule. In order to further assess the fine specificity of the binding of DE8 to HD_{11}-coated microtiter wells, we also tested for the ability of the metabolites to inhibit this binding. In this assay various concentrations of T-2 or T-2 metabolites were added to HD_{11}-coated microtiter wells. A concentration of DE8-b which gave approximately 50% binding was then added to the wells and reactivity was developed by avidin-HRP and the appropriate substrate. The results shown in Table 4 revealed a similar inhibition pattern obtained with that of the binding of HD_{11} to T-2 (Table 3). Taken together, these results suggested that the T-2 binding site of HD_{11} is similar to the DE8 binding site of HD_{11}. We also assessed the ability of T-2 metabolites to inhibit the binding site of HD_{11}-b to DE8- coated microtiter wells (data not shown). The results showed the same pattern of inhibition; metabolites that inhibited the binding of HD_{11} to T-2 also inhibited the of binding of DE8 to HD_{11} and the binding HD_{11} to DE8. On the other hand, metabolites that did not inhibit the binding of HD_{11} to T-2 had no effect on the binding of DE8 to HD_{11} or HD_{11} to DE8.

These results taken together suggest that DE8 mAb recognized the T-2-binding site of HD11. Although direct evidence is lacking, the results do suggest that DE8 may be classified as an $Ab2_b$ anti-Id which expresses the internal image of the antigen (e.g., it mimics the three-dimensional conformation of the antigen).

Induction of Circulating Anti-T-2 Antibody in BALB/c Mice by Injection of DE8 Antibody

To assess the ability of DE8 anti-Id antibody to induce a circulating anti-T-2 immune response, 3 groups of 5 BALB/c mice each received the following immunizations:

1. T-2-ovalbumin
2. Unconjugated DE8 anti-Id precipitated in alum
3. De8-KLH conjugate precipitated in alum

The first injection was given intradermally in Freund's complete adjuvant, followed by a boost in Freund's incomplete adjuvant. Subsequent boosts were done with antigens in saline intraperitoneally. All mice received by-weekly injections. The immunized mice were bled subsequently and the sera assayed

for anti-T-2 antibodies and for the expression of the idiotype on the original Ab-1 preparation (HD_{11}). Figure 11 shows the anti-T-2 reactivity of the sera of groups of five mice after the third injection of T-2-OVA, DE8-alum or DE8-KLH-alum, as assessed by an ELISA employing T-2-BSA-coated microtiter wells (16). The results show that all 3 groups of mice made significant anti-T-2 antibodies following the third injection of antigens. It is interesting to note that the group of mice that received DE8-KLH-alum produced the highest titer of anti-T-2 antibodies, followed by the mice immunized with DE8-alum and T-2 OVA. These results confirm our previous observations that conjugation of an Abl to a protein carrier such as KLH induces a significantly higher immune response in a syngeneic system than unconjugated Abl antibody. In order to assess the level of expression of HD_{11} Id^+ anti-T-2 in the 3 groups of mice, the following ELISA was utilized. Microtiter wells were coated with purified HD_{11} anti-T-2 antibody overnight at 4°C. After blocking of non-specific sites, mixtures of a dilution of DE8-biotin giving approximately 50% binding to HD_{11} with various dilutions of mouse sera were added to the microtiter wells and incubated for 1 hr at 37°C. After washing off unbound antibodies, DE8-b bound to HD_{11}-coated wells were detected by the appropriate substrate. The presence of HD_{11} (HD_{11}-like) anti-T-2 antibodies in the mouse sera will inhibit the binding of DE8-b to HD_{11}-coated wells. The results are shown in Figures 12 and 13 for DE8-KLH-alum and DE8-alum immunized mice, respectively. Although the difference is not significant, it generally appears that DE8-KLH-alum immunized mice produced a somewhat higher level of HD_{11}^+ anti-T-2 antibodies. On the other hand, mice immunized with T-2-OVA did not produce significant levels of HD_{11} Id^+ antibodies. The range of inhibition of the binding of DE8-b to HD_{11}-coated wells by sera from mice immunized with T-2-OVA at 1:10 dilution ranged from 0 to 19.3% (data not shown). Thus, these data clearly demonstrated the ability to modulate an immune response by anti-Id antibodies to contain a particular Id^+-antibody, in this case HD_{11} anti-T-2 antibody, which we have shown earlier to protect against T-2 <u>in vitro</u> cytotoxicity. This represents a major advantage of the anti-Id based vaccine against the toxins under investigation in this contract, namely the anti-Id ability to modulate the anti-toxin antibody response to contain primarily anti-toxin antibodies which are protective, a situation not obtained when toxin is used as the immunogen.

To assess whether the anti-T-2 antibodies in the DE8-KLH-alum, DE8-alum, or T-2-OVA immunized mice could protect against Hep-2 cytotoxicity induced by T-2 toxins, various dilutions of mouse sera were added to a known concentration of incorporation of the radioactive amino acid was determined by scintillation counting as described previously. The results in Figure 14 indicated that sera from mice immunized with DE8-KLH-alum or DE8-alum were effective in protecting Hep-2 cells against T-2 toxin-induced cytotoxicity, whereas sera from mice immunized with T-2-OVA were not as effective in protecting against T-2 cytotoxicity. The specificity of the anti-T-2 antibody response in all three groups of mice was demonstrated by the fact that their binding to T-2 was completely inhibited by 50 ul of a solution of T-2 BSA at 100 ug/ml (data not shown). BSA itself did not inhibit this binding. Interestingly, free T-2 toxin was not capable of inhibiting the binding of the anti-T-2 mouse sera to T-2-BSA.

In summary, injection of DE8 anti-Id either unconjugated or conjugated to KLH results in the induction of a specific anti-T-2 antibody response comprising primarily of HD11-like or $HD11^+$ anti-T-2 antibodies.

DE8 Induce A Protective Anti-T-2 Antibody Response In-Vivo

To assess whether this DE8-induced anti-T-2 antibody response is protective against the *in vivo* toxicity to T-2, groups of BALB/c mice immunized with 4 injections of DE8-KLH or control anti-Id mAb (anti-anti-hepatitis B surface antigen, a gift from Dr. R. Kennedy, Southwest Foundation for Biomedical Research, San Antonio, TX) were challenged intradermally with various doses of T-2. The results are shown in Table 5. Immunization of mice with DE8 anti-Id conjugated to KLH completely protected the mice against the *in vivo* toxicity of T-2. In our hands, the LD_{50} of T-2 in normal mice is approximately 5 mg/kg body weight. Even at a dose of 20 mg/kg, all five DE8-treated mice were protected against T-2 toxicity. One mouse in the group that received 10 mg/kg body weight died, possibly because of a low anti-T-2 antibody response.

We are immunizing another group of 30 BALB/c mice with DE8-KLH in order to confirm these results. WE will also increase the T-2 doses to assess the degree of protection. These results represent, in our knowledge, the first demonstration of a successful anti-Id based vaccine against a small molecular weight biological toxin, and land support to the notion of the usefulness of anti-Id as vaccines in toxicology where the very toxic nature of the toxins preclude their use as immunogens.

B. The Sodium Channel Blocker System.

1. Tetrodotoxin.

The following represents the results of our attempt to produce high binding affinity monoclonal anti-tetrodotoxin (TDT) antibodies. Although a large number of anti-TDT mAbs have been generated during the contract, this section will concentrate on detailing results obtained with two anti-TDT mAbs with the highest binding affinity, namely TD2C5 and TD13a1.

Female BALB/c mice (6-8 weeks old) were immunized at weekly intervals with 50 ul containing 50 ug of TDT-KLH conjugate per mouse mixed with an equivalent volume of Freund's complete adjuvant (i.d., for the first immunization, Freund's incomplete (i.d.) for the second, and PBS (i.p.) for all subsequent injections. TDT was conjugated to keyhole limpet hemocyanin via formaldehyde as the coupling agent in order to render it immunogenic. Briefly, 300 ug of TDT in 100 ul of 1 M sodium acetate buffer, pH 7.6, was conjugated to 80 ul of KLH (53 mg/ml) in the presence of 60 ul of 37% formaldehyde. The mixture was shaken at 37°C for 3 days and dialyzed for 24 hr at 4°C. Following dialysis, the TDT-KLH was precipitated with 800 ul of 10% aluminum potassium sulfate and 500 ul of 1 N NaOH at 4°C. The resulting precipitate was washed six times with cold PBS. Conjugates were prepared freshly for each immunization. Sera were collected at weekly intervals. Four days after a final i.v. immunization in the tail vein, the spleen was removed and the cells fused with NS-1 myeloma cells in 45% polyethylene glycol-4000 at a ratio of three to six spleen cells per myeloma cell in serum-free RPMI. The fused cells were cultured in 96-well microculture plates at a density of 0.5 to 1 x 10^5 cells/ml in the presence of RPMI-HAT selective medium containing 10% fetal cell serum (FBS), 2 mM glutamine, 1x non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, penicillin (50 IU/ml) and streptomycin (50 ug/ml). Plates were refed with HAT medium 3 days later and subsequent feedings contained HT but no aminopterin.

Culture supernatants were screened for TDT-specific antibodies by ELISA (17). Briefly, 700 ug of TDT or saxitoxin (STX) obtained from Dr. John Hewetson (USARMDC, Fort Deterick, MD) were conjugated to 6 mg of BSA with 41 ul of 37% formaldehyde and incubated for 3 days at 37°C. They were then dialyzed for 3 days at 4°C. against PBS. Polystyrene plates were coated with 50 ul/wells of a 10 ug/ml solution of STX-BSA or TDT-BSA and stored at 4°C until needed. The plates were blocked with 200 ul of 5% normal goat serum in PBS for 1 hr at 37°C followed by incubation with supernatants (100 ul) for an additional hour at pH 7.5, 50 ul of a 1:2500 dilution of goat anti-mouse Ig conjugated to horseradish peroxidase, were then added for 1 hr at 37°C. The wells were again washed and were then developed with 100 ul of ABTS [2,2-azino-di-3-ethyl-benzthiazoline-6-sulfonate, 0.3% in 0.1 M citric acid buffer, pH 4.0]. The reaction was terminated by adding 100 ul of 5% SDS in PBS and absorbance read at 410 nm in a Dynatech microplate reader.

Positive clones were transferred to 24-well Costar plates and subclones by limiting dilution as rapidly as possible. Positive subclones were selected on the basis of antibody reactivity by ELISA and were resubcloned until the majority of the subclones were antibody-positive. Aliquots of positive clones were cryopreserved in liquid N_2.

Of a total of 9,329 clones tested, 337 clones (3.6%) were positive in a binding ELISA employing TDT-BSA coated microtiter wells. Among these, two highly stable clones, designated TD2C5 and TD13a1, were isolated (17). The specificity of these two anti-TDT clones for TDT in a competitive inhibition ELISA employing TDT-BSA-coated plates is shown in Table 6. Addition of 100 ug/ml of free TDT to the anti-TDT antibodies completely inhibited the binding of both antibodies to TDT-BSA, whereas the equivalent amounts of STX or PBS had no effect on the binding. Free TDT at concentration of 50 ng/ml inhibited the binding of TD2C5 and TD13a1 mAbs by approximately 50% and 25%, respectively. Neither mAbs bound to STX-BSA-coated microtiter wells, further attesting to their binding specificity against TDT (data not shown). Both mAbs were determined to be of the $IgG_{1,k}$ subclass both by direct immunofluorescence staining of hybrid cells and by a commercial isotyping kit.

The relative binding affinity of the mAbs were determined by IC_{50} competitive inhibition enzyme immunoassay (18). Dilutions of antibody concentrations were tested for binding to TDT-BSA-coated plates and the 50% binding end-point estimated. The concentration yielding 50% binding was incubated with an equivalent volume containing various concentrations of TDT ranging from 12 ng/ml to 50 ug/ml, in two-fold dilutions for 1 hr at 37°C followed by addition to TDT-BSA-coated plates. To determine the IC_{50}, or the molar concentration of toxin giving 50% reduction in optical density, the optical densities were converted to percent reduction of maximal optimal density (y). The y values were then converted to a logit according to the following formula:

$$\text{Logit} = \ln \frac{y}{100 - y}$$

Logit values were plotted against molar TDT concentrations on semi-log paper and the IC_{50} was determined graphically by dropping a vertical line to the X axis from the point where the straight line crossed logit at 0. The relative affinity constant K_a is the reciprocal of the IC_{50}

Figure 15 shows the titration inhibition data for mAb TD2C5. A predetermined concentration of TD2C5 giving approximately 50% binding in a TDT-BSA ELISA was admixed with concentrations of TDT ranging from 10^{-4} M to 10^{-8} M. The reduction in TD2C5 binding in the presence of TDT is shown in Figure 15A. The binding of TD2C5 was inhibited by approximately 40% in there presence of as little at 10^{-7} M solution of TDT, whereas a 1000-fold higher concentration of STX had no effect on the binding. The determination of TD2C5 IC_{50} is displayed in Figure 15B, and was generated from the data presented in Figure 13A, yielding an IC_{50} value of 2×10^{-7} M. The K_a value is therefore the reciprocal of the IC_{50} or 0.5×10^{7} M^{-1}. Similar analysis was employed with mAb TD13a1 which has a K_a value of 0.2×10^{7} M^{-1} (data not shown).

To assess for the potential protective effects of TD2C5 and TD13a1 mAbs, two assays were used. The <u>in vitro</u> brain membrane receptor binding assay was used to test the ability of the mAbs to inhibit the binding of [11-^{3}H] STX to rat brain cell membrane, and the <u>in-situ</u> nerve response amplitude was employed two assays were performed as follows:

<u>In vitro membrane receptor binding assay.</u> The ability of STX and TDT to bind stoichiometrically to a common receptor site was employed in radio-receptor assays to demonstrate the protective abilities of anti-TDT monoclonal antibodies. Crude membrane receptors from the brains of female AXC/SSh rates (from the colony maintained at the Southwest Foundation for Biomedical Research) were prepared according to Strichartz (19) and their protein content determined by the Bradford method using a commercially-available kit. Aliquots were stored at -80°C until needed. Optimum incubation times and binding conditions of [^{3}H] STX to the brain membranes were determined according to the radioreceptor studies above. Because the TDT specificity of the anti-TDT antibodies precluded their binding to [^{3}H] STX antibodies. Membranes (500 ug protein in 25 ul) were added to microfuge tubes and incubated under saturating conditions in the presence of [11-^{3}H] STX with a sp. act. of 63 Ci/mmol at a final concentration of 6nM. Various amounts of radioinert TDT (0.1 ng to 10ng) were included in the presence and absence of a constant, known, concentration of antibody for 1 hr at 4°C and the amount of [^{3}H] STX bound measures. Two competition curves for [^{3}H] STX binding were thus generated , with and without anti-TDT antibody, respectively, and the amount of TDT giving 50% of [^{3}H] STX binding in the presence and absence of antibody calculated graphically on each curve. The difference between these two values represented the amount of TDT bound by antibody. Similar displacement curves were generated in the presence of radioinert STX in order to demonstrate the specificity of binding of anti-TDT antibodies for TDT and not STX.

<u>In situ protection of peripheral nerve function with anti-TDT antibody.</u> Thirty male Sprague-Dawley rate (age 40-60 days) were used. Sodium pentobarbital (Nembutal) was administered intraperitoneally (60 mg/kg body weight) and the rats were placed on an isothermal heating pad. The left rear leg and hep were shaved and the leg was immobilized on a small surgical platform. A dissection was performed to expose the sciatic nerve and tibial branch entering the gastrocnemius muscle. The skin surrounding the incision was lifted and secured with hemostats to form an enclosure for bathing the nerve in warm 0.9% NaCl that contained the various antibody and TDT concentrations. The solutions were applied and withdrawn using pasteur pipettes.

Bipolar stimulating and recording electrodes manufactured from teflon insulated silver wire were positioned beneath the sciatic and tibial nerves, respectively. Single, square wave pulses (0.05 msec duration) were used to evoke action potentials which were recorded with a WPI differential amplifier. The tibial nerve responses were displayed and photographed using a Tektronix storage oscilloscope. Response amplitude was measured as the difference between the most positive and negative deflection of the compound action potential waveform. The stimulus intensity (3 to 6 volts) was adjusted to produce maximal compound action potential amplitude. Prior to any application of antibody or toxin, the action potential amplitude was recording every 30 sec for a 3 min period and the average of these six responses formed the Baseline data for each preparation. Experimental treatment consisted of soaking the nerve for two 5 min periods in either antibody alone (Control), TDT alone, or TDT combined with added to the toxin solution 1 hr prior to application. AFter 5 min of soaking the nerve the solution was then removed and the average response amplitude was determined as described above for Baseline data. The same solution was then reapplied for an additional 5 min, withdrawn, and the average response amplitude was again determined. At the end of the experiment the animal was sacrificed with an overdose of Nembutal.

Baseline and experimental responses were compared using analysis of variance (ANOVA) with a general linear model procedure (SAS Institute, Cary, NC). Data displaying an overall significance level of $p < 0.05$ were further analyzed with the Newman-Keuls multiple range test.

Using the brain receptor displacement assay, both anti-TDT mAbs were able to bind to radioinert TDT, preventing its binding to the brain membrane receptors which resulted in an increased binding of [^{3}H] STX to these receptors (Fig. 16). TD2C5 mAb at 1.3 ug was able to displace approximately 5 ng of TDT at the 50% inhibition point. In terms of molar capacity, 1 M or TD2C5 bound 1.9 M of TDT which approached the theoretical capacity of 2 M of antigen bound by 1 M of an IgG antibody. The specificity of TD2C5 was also reflected in the inability of even a 10-fold higher (13.0 ug) of TD2C5 to bind STX. Similar assays were performed with Td13a1 which bound 0.87 M of TDT/M of antibody.

The results of the <u>in-situ</u> protection against TDT-induced nerve toxicity by TD2C5 are presented in Table 7. The amplitudes of the action potentials generated in rate tibial nerve were recorded in the presence of 5.5 uM of Td2C5 alone (control), in the presence of 10 uM TDT alone (TDT), and in the presence of 10 uM TDT plus 5.5 uM TD2C5 (Exp. 1), 10uM TDT plus 2.78 uM TD2C5 (Exp. 2) or 10 uM TDT plus 0.5 uM TD2C5 (Exp. 3). Baseline recordings were obtained before addition of any compound and effect of the test agents were measured after 5 min and 10 min exposure periods. TD2C5 applied alone did not have any significant effect in the amplitude of the response. Application of TDT alone resulted in a reduction of the response amplitude by 37% of baseline response after 5 min and 22% after 10 min. Addition of TD2C5 resulted in a dose-dependent restoration of the response amplitude diminished by TDT. The two highest concentrations of TD2C5 (5.5 uM and 2.78 uM) significantly counteracted the diminution of the response amplitude, whereas addition of 0.5 uM TD2C5 afforded little protection against TDT toxicity. Typical effects of TDT on action potentials in the presence of 5.5 uM TD2C5 alone (control), 10 uM TDT alone (TDT), and 10 uM TDT plus 5.5 uM TD2C5 (TD2C5) are depicted in Figure 17. The small differences in waveform shapes shown in the three baseline traces were due to slight variations in electrode placement that

normally occur from one recording preparation to another and do not reflect any significant differences in the nerve response. Similar results were obtained with TD13a1, however the protection provided by this mAb was not as pronounced as that of TD2C5 (data not shown). This is not surprising considering the slightly lower K_a of TD13a1 compared to that of TD2C5.

To our knowledge, TD2C5 and TD13a1 mAbs represent the first documented monoclonal anti-TDT antibodies which effectively inhibit the binding of toxin to rat brain cell membrane and provide significant protection against nerve toxicity induced by TDT. It is not unreasonable to speculate that these mAbs, especially TD2C5, may provide passive protection against the in-vivo toxicity of TDT. This is under investigation. Furthermore, our success in producing an anti-Id mAb against HD11 anti-T-2 (discussed above) which was capable of inducing a protective systemic anti-T-2 antibody response lends support to our continued effort to generate anti-Id specific for TD2C5 which may be useful as vaccines against TDT toxicity. Approximately three fusions (about 950 clones screened) have been performed with spleen cells from mice immunized with TD2C5-KLH. Up to date, no antigen-inhibitable anti-Id mAbs (Ab_{2b} class) have been isolated, although two mAbs were generated which were not inhibited by TDT in their binding to TD2C5, but were specific in their binding to TD2C5 suggesting their classification as Ab_{2a} in nature.

2. Saxitoxin.

As is the case with anti-TDT mAbs described above, our research work with saxitoxin (STX) has resulted in a number of mAbs specific in their binding to STX. However, the majority of the mAbs are of a relatively low affinity of binding. The following section describes the two monoclonal anti-STX with the highest binding affinities.

The methodology employed in the STX work is similar to that described above for TDT. STX (from Dr. John Hewetson, USAMRIID) was conjugated to KLH for mouse immunization essentially as described for TDT-KLH. The fusions performed with spleen cells from mice immunized with STX-KLH, the screening for anti-STX mAbs, the determination of relative binding affinity constants and the in-situ protection experiment using rat sciatic nerve are essentially as described for TDT. The in-vivo rat cell membrane receptor binding assay was performed with the following modification Since [^{3}H] STX is available commercially, the ability of the anti-STX mAbs to inhibit the binding competition curves, membranes at 500 ug/0.025 ml were added to microfuge tunes and incubated with [11-^{3}H] STX with a specific activity of 63 Ci/mM at a final concentration of 6nM and various amounts (0.1-10 ng) of unlabeled STX. As more radio inert STX is added, there is less labeled STX binding in this competition membrane receptor binding assay. To determine anti-STX antibody activity, the assay was performed in the presence or absence of a constant and known amount of antibody for 1 hr at 4°C and the amount of [^{3}H] STX bound was measured. Two competition curves for labeled STX binding were generated with and without antibody, respectively. The amount of STX giving 50% of labeled STX binding in the presence and absence of antibody was calculated graphically on each curve. The difference between these two values represented the amount of STX bound by antibody, and thus unavailable for competing with [^{3}H] STX binding to rat brain cell membrane.

A total of 6,994 hybrids from 10 separate fusions of the STX-KLH-immunized mice were screened by STX-BSA ELISA, of which 148 (2.1%) showed binding to STX (20). Antibody specificity was further demonstrated by a competitive ELISA utilizing both STX and TDT. The results are shown in Table 8. Two highly stable clones, termed S1A5 and S3E.2, were isolated that were shown to be specific for STX. Preincubation of 100 ug/ml of STX with either mAb completely inhibited their binding to STX-BSA, whereas the same amount of TDT had no significant effect. Free STX at a concentration of 500 ug/ml inhibited the binding of S1A5 and S3E.2 mAbs by 63% and 39%, respectively. In addition, neither mAb bound to TDT-BSA-coated microtiter wells, further attesting to the specificity of the mAbs for STX and not TDT (data not shown). The isotypes of S1A5 and S3E.2 mAbs, as determined by direct immunofluorescence and ELISA as mentioned above, were of the IgM_k and IG_{1k} subclass, respectively. The binding affinity constants of the two mAbs were determined using the IC_{50} logit analysis as described for anti-TDT mAbs. The titration-inhibition data for S3E.2 are shown in Figure 18. The binding of S3E.2 in an STX-BSA ELISA was inhibited by 25% by as little as 5×10^{-7}M solution of STX, whereas a 500-fold higher concentration of TDT had no effect (Fig.16A). The IC_{50} analysis of S3E.2 mAb is displayed in Figure 16B and was generated from the data from Figure 16A, yielding an IC_{50} or 0.9×10^6 M^{-1}. By similar analysis, the K_a value for S1A5 was 0.5×10^6 M^{-1} (data not shown).

In the rat brain membrane displacement assay, both S3E.2 and S1A5 anti-STX mAbs demonstrated the ability to bind STX and effectively prevent its binding to the brain receptor. SE3.2 (5.64 ug) was able to displace 0.89 ng (1.3-0.4 ng) of STX at the 50% inhibition and point (Fig.19). In molar terms, 1 M of S3E.2 mAb displaced 0.09 M of STX. Similar analysis shown 1 M S1A5 to displace only 0.005 M STX (data not shown). These mAbs were next examined for their ability to protect against STX-induced nerve toxicity <u>in-situ</u>. The results are summarized in Table 9. When 10 uM of either S3E.2 or S1A5 mAbs were applied alone to the exposed sciatic nerve, there was no significant changes in the response amplitudes observed (Table 9 and Figure 20). Application of STX alone reduced the response amplitudes to 28% and 15% of baseline after 5 and 10 min, respectively, in the S1A5 set of experiments, and to 38% and 9% of baseline, respectively, for the S3E.2 set of experiments. Neither 5 uM nor 10 uM of S1A5 provided any significant protection against STX toxicity (Table 9). One the other hand, a dose-dependent protection was obtained with S3E.2 mAb, with 30 uM concentration of S3E.2 providing the most significant protection. However, by 10 min the protective ability of all concentrations of S3E.2 mAb over time was presumably due to the establishment of a new equilibrium in STX binding between the lower STX affinity, of S3E.2 (K_a of 10^6 M^{-1}) and the higher STX affinity of nerve (K_a of 10^9 M^{-1}).

Two monoclonal anti-STX of high specificity of STX have been isolated. The S3E.2 represents to our best knowledge the first reported anti-STX mAb to protect against STX <u>in-situ</u>. The S1A5 anti-STX, although highly specific for STX, did not appear to have any protective ability. Whether this lack of protection results from S1A5 lower K_a, its IgM isotype or recognition of STX epitope not involved in toxicity is not known. However, the ability of these mAbs to inhibit the binding of STX to rat brain membrane receptors suggests a potential protective effects of these mAbs, especially S3E.2, both <u>in-vitro</u> and <u>in-vivo</u>.

3. Attempts to Increase Affinity Constants.

As mentioned previously, the generation of high affinity mAbs that would effectively compete for toxin binding represents a major challenge in this research project. Some of the experimental approaches designed to increase the affinity constants being investigated are as follows:

a) Use of new conjugate as immunogen (toxin-SPDP-carrier)
b) Small doses of immunogens (Toxin-formaldehyde-carrier)
c) Boosting toxin-hyperimmune mice with sublethal doses of toxin
d) In-vitro stimulation of spleen cells from hyperimmune mice with free toxin.

Because mAbs with specificity for STX in general represent the ones with the lowest binding affinity constants, our efforts in increasing affinity of binding have centered around the STX project.

a) The first approach involves the development of a potentially highly immunogenic STX conjugate by coupling STX to KLH via the linker N-succinimidyl 3-2 (pyridyldithio) propionate (SPDP). The propionic acid linker is thought to enhance immunogenicity by making STX more exposed and accessible for immune recognition. To prepare the conjugate, 50 mg of KLH in 15 ml of borate buffered saline (BBS, pH 7.25) was added to 5.2 mg of SPDP and 200 ul dimethyl formamide (1000:1 molar excess). The mixture was reacted for 1 hour at room temperature and dialyzed overnight against BBS, pH 8.0, at 4°C. To the KLH-SPDP) was added 3.85 mg of dithiothreitol in BBS. The mixture was dialyzed against 2 l of BBS containing 3 mM sodium borohydride for 1 hr at 4°C. The buffer was changed and dialysis performed against BBS for another hour at 4°C. The STX derivative was then added to the dialysate. The derivative was prepared by drying 5 mg STX under vacuum followed by heating at 110°C for 3 hr in the presence of 7.5 N HCL. The resulting decarbamoyl-STX was dried under vacuum. The STX-SPDP-KLH conjugate was reacted for 3 hr at room temperature and dialyzed overnight. The dialysis buffer was changed 2 times during the next 2 days. Binding ELISA assay indicated the presence of STX on the prepared conjugate although the exact amount present is not known. Three BALB/c mice were immunized with this newly prepared STX-KLH conjugate. After three injections of this new STX-KLH conjugate, significant anti-STX titer could be detected in the mouse sera by binding ELISA to microtiter wells pre-coated with STX-BSA conjugated by the formaldehyde method described above (data not shown). Two separate fusions have been performed with spleen cells from mice immunized with STX-SPDP-KLH. Up to date, we have not been successful in isolating anti-STX mAbs with higher affinity of binding than those of S3E.2 and S1A5 mAbs.

b) Another approach being attempted involves the immunization of mice with small doses (1.0 ug/mouse vs. 50 ug/mouse) of STX-KLH conjugated either by the formaldehyde or SPDP methods at monthly intervals instead of two-week intervals. It is possible to increase the affinity constants of the immune response with low doses of antigens presumably as a result of a preference in the binding and subsequent stimulation, proliferation and maturation of B lymphocytes with high affinity surface immunoglobulins to a limited amount of circulating antigens. The effectiveness of this approach is under investigation.

c) The third approach which is similar to the approach just described involves boosting hyper-immune mice in-vivo with sublethal dose of free toxin. BALB/c mice hyperimmunized with STX-KLH either conjugated by the formaldehyde or SPDP method were boosted with 5 uM of free STX, a sublethal dose of STX. As in the above approach, it is hoped that circulating B lymphocytes with high affinity surface immunoglobulins will seek out and preferentially bind to the limiting amount of circulating free STX.

d) The last approach involves as in-vitro secondary immunization of STX-primed mouse spleen cells prior to fusion. Spleen cells from STX-KLH immunized mice were cultured in the presence of RPMI 1640 with 10% fetal bovine serum (FBS), 20% allogeneic supernatant and 1 uM STX. Allogeneic medium was prepared by co-culturing dissociated spleen cells from two histo-incompatible mice. Spleen cells from BALB/c and C57Bl at a density of 10^6 cells/ml were mixed at a ratio of 1:1 in 10% serum-containing RPMI 1640 medium for 3 days. The in vitro secondary immunization culture was carried out at a density of 5×10^6 cells/ml for 4 days. Cells were then harvested by centrifugation and fused to NS-1 mouse myeloma cells. The percentage of positive anti-STX hybrids was considerably enhanced by the in vitro secondary immunization technique ranging from 12.3 to 20.6%, as compared to approximately 2-4% from a fusion without in vitro boost. The increased percentages presumably reflect selection for anti-STX reactive spleen cells and their expansion during the secondary immunization culture period. Although a number of these clones demonstrated specific reactivity to STX and not tetrodotoxin (TDT), IC_{50} analysis of the antibody-containing supernatants of these cultures, to date, has not revealed any of these to have strong binding affinities to STX.

Although no success in enhancing the affinity constants of the generated mAbs specific for STX has been achieved with the limited number of experiments conducted so far, we would like to continue our efforts along these areas in systematically exploring these approaches in order to achieve enhanced affinity constants.

SUMMARY

In summary, we have demonstrated the feasibility of using the anti-Id-based approach in the development of a successful vaccine against a small molecular weight, nonproteinaceous and highly toxic biological compound, namely mycotoxin T-2. This represents the first demonstration of an antibody-based vaccine against this class of biological toxins. It is of interest to note the ability of the anti-Id antibody to modulate the immune response toward the expression of a specific Id determinant (HD11 Id), which in this case is associated with protection against toxicity. HD11 represents the only protective anti-T-2 mAb among the approximately 50 mAbs generated in our laboratory with T-2 binding activity. Thus, not only is the anti-Id-based vaccine approach the only practical approach when dealing with potent biological toxins, this approach was also shown in this instance to provide better protection against toxicity than immunization with the native toxic conjugated to protein carriers, as a result of modulation of the antibody response toward the production of antibodies directed against protective epitopes. It is anticipated that successful anti-Id based vaccines can also be developed for saxitoxin and tetrodotoxin, in particular, and for other small molecular weight biological toxins in general.

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FIGURE LEGENDS

Fig. 1. HD_{11} mAb binding to T-2-BSA-coated microtiter wells. Each value represents the mean OD_{410nm} ± S.E. of triplicate determinations.

Fig. 2. Inhibition of HD_{11} mAb binding T-2-BSA-coated plates. Each value represents the mean of $O.D._{410nm}$ + S.E. of triplicate determinations.

Fig. 3. T-2-cytotoxicity Hep-2 KB human cell lines. Each point represent the mean cpm ± S.E. of triplicate determinations.

Fig. 4. Specificity of rabbit anti-Id binding HD_{11} mAb.

Fig. 5. Inhibition of HD_{11} mAb binding T-2-BSA-coated plates by rabbit anti-Id.

Fig. 6. Binding of DE8-b mAb to HD_{11} - coated wells. DE8-b (■); Control anti-Id-b (●).

Fig. 7. Inhibition of HD_{11}-b binding to T-2-BSA-coated wells by DE8 mAb. DE8 mAb (■); control mAb (●).

Fig. 8. Free T-2 inhibits the binding of HD_{11}-b to DE8-coated plates. T-2 mycotoxin (■); TDT (●).

Fig. 9. Free T-2 inhibits the binding of DE8-b to HD_{11}-coated plates.

Fig. 10. DE8 abrogates the protective effect of HD_{11} against T-2 cytotoxicity. DE8 (■), and control anti-Id (●). The mean cpm ± SD of cultures with and without T-2 were 58,156 ± 3,214 and 140,660 ± 1,101 cpm, respectively. The mean cpm ± SD of cultures with T-2 and 100 ug/ml of HD_{11} was 138,747 ± 9,341 cpm.

Fig. 11. Anti-T-2 reactivity of groups of BALB/C mice immunized with DE8-KLH (●); DE8 (■), and T-2-OVA (▲). Each value represents mean $O.D._{410nm}$ of 5 BALB/C mice ± S.E.

Fig. 12. Inhibition of DE8-b binding to HD_{11} - coated plates by DE8-KLH-immunized mouse sera.

Fig. 13. Inhibition of DE8-b binding to HD_{11} - coated plated by DE8-immunized mouse sera.

Fig. 14. Serum from mice immunized with DE8 abolishes the protective effect of HD_{11} against T-2 cytotoxicity of the Hep-2 cell line. DE8-KLH immune (●); DE8-immune (■); T-2-OVA-immune (▲); and normal serum (◆).

Fig. 15. Inhibition of S3E.2 anti-STX antibody binding by STX. The mAb, S3E.2 (panel A) was incubated in the presence or absence of STX (●) or TDT (●) at the concentrations indicated. The IC_{50} determination of the data presented in panel A is depicted in panel B.

Fig. 16. Competitive displacement of [^{3}H]STX binding to rat brain membranes by S3E.2. [^{3}H] binding was performed in the presence of STX alone (●) or STX plus 5.64 ug of S3E.2 (o) for 1 hr at 4°C. The broken line represents the 50% inhibition of binding in the presence of S3E.2.

Fig. 17. Comparison of tibial nerve action potentials in the presence or absence of S3E.2 antibody. Baseline recordings were made prior to the addition of antibody or STX. Measurements were again performed 5 and 10 min following addition of 10 uM S3E.2 alone (control), 1 uM STX plus 30 uM S3E.2. The vertical bar and horizontal bar in the lower right hand corner denote 1 millivolt and 1 second, respectively.

Fig. 18. Inhibition of TD2C5 anti-TDT antibody binding by TDT. The mAb, TD2C5 (panel A) was incubated in the presence or absence of STX (o) or TDT (o) at the concentrations indicated. The IC_{50} determination of the data presented in panel A is depicted in panel B.

Fig. 19. Competitive displacement of STX- and TDT-mediated inhibition of [^{3}H] STX binding to rat brain membranes by TD2C5. [^{3}H] STX binding was performed in the presence of: (o) STX alone, (▲) TDT alone, (●) STX plus 13 ug TD2C5, and (▲) TDT plus 1.3 ug TD2C5 for 1 hr at 4°C.

Fig. 20. Comparison of tibial nerve action potentials in the presence or absence of TD2C5 antibody. Baseline recordings were made prior to the addition of antibody or TDT. Measurements were again performed 5 and 10 min following addition of 5.5 uM TD2C5 alone (control), 10 uM TDT alone (TDT), or 10 uM TDT plus 5.5 uM TD2C5 (TD2C5).

Publications

Abstracts

Huot, R.I., Castro, J.A., and Chanh, T.C. Monoclonal antibodies specifically inhibit binding of sodium channel blockers to rat brain membranes, Fed. Am. Soc. Exp. Biol. 2(4):A678, 1988.

Chanh, T.C., Reed, R.C., Frenzel, G., and Huot, R.I. Anti-idiotypic antibodies against monoclonal antibody specific for the trichothecene mycotoxin T-2. Fed., Am. Soc. Exp. Biol. 2(4):A677, 1988.

Huot, R.I., J.A., Castro, D.L. Armstrong and T.C. Chanh. Polyclonal rabbit anti-idiotypic antibodies against a protective monoclonal antibody specific for the sodium channel blocker Tetrodotoxin. Sixth An. Texas Immunol. Conf. Galveston, TX, 1988.

Schick, M., Hewetson, J., Nabers, P., and Chanh, T.C. Monoclonal anti-idiotype antibody as vaccine against mycotoxin (T-2)-induced cytotoxicity. Fed. Am. Soc. Exp. Biol. 3:A1135, 1989.

Manuscripts

1. Huot, R.I., Armstrong, D.L., and Chanh, T.C. In vitro and in situ inhibition of the sodium channel blocker saxitoxin by monoclonal antibodies. J. Toxicol. Environ. Health. 27:281-393, 1989.

2. Huot, R.I., Armstrong, D.L., Hewetson, J.H., and Chanh, T.C. Monoclonal antibodies inhibit the sodium channel blocker tetrodotoxin in vitro and in situ, J. Clin. Invest. 83:1821-1826, 1989.

3. Chanh, T.C., Huot, R.I., Schick, M.R., and Hewetson, J.H. Anti-idiotypic antibodies against a monoclonal antibody specific for the trichothecene mycotoxin T-2. Toxicol. Appl. Pharmacol., 100:201-207, 1989.

4. Chanh, T.C., G. Rappocciolo, and J.F. Hewetson. Monoclonal Anti-Idiotype induces a protective antibody response against the trichothecene mycotoxin T-2. In press J. Immunol., June 1990.

5. Chanh, T.C., and J.F. Hewetson. Fine specificity of a monoclonal anti-T-2 mycotoxin antibody. Submitted.

TABLE 1

Protection against *in vitro* T-2 cytotoxicity by monoclonal antibody HD_{11}

Antibody (μg/ml)	Hep-2 cells			KB cells		
	- T-2	+ T-2[a]	% I	- T-2	+ T-2[b]	% I
None	403,412 ± 21,512[c]	151,007 ± 8,080	62.3[d]	298,387 ± 18,151	72,304 ± 6,819	75.8
HD_{11} (100)	463,724 ± 32,010	442,363 ± 25,905	4.6	309,240 ± 25,156	337,034 ± 28,816	0
HD_{11} (10)	436,929 ± 22,508	428,426 ± 21,115	1.9	299,334 ± 19,815	298,164 ± 19,150	0.4
HD_{11} (1.0)	419,008 ± 19,052	403,769 ± 18,456	3.6	278,158 ± 21,201	189,981 ± 12,100	31.7
HD_{11} (0.1)	429,342 ± 18,918	298,133 ± 15,615	30.5	301,961 ± 28,142	75,005 ± 7,015	75.1
HD_{11} (0.01)	415,905 ± 20,015	210,293 ± 16,518	49.4	311,479 ± 21,560	64,583 ± 8,001	79.2
Control IgG_1 (100)	389,641 ± 29,156	148,150 ± 10,108	61.9	286,168 ± 19,158	75,026 ± 7,105	73.8

[a] 16 ng/ml T-2 was used in Hep-2 cell assays.

[b] 10 ng/ml of T-2 was used in KB cell assays.

[c] Mean of triplicate cpm ± S.E.

[d] Mean percent inhibition of [^{3}H]leucine uptake.

TABLE 2
Effects of Antibody Pretreatment on LD_{50} and Survival Time

BALB/c Mice Pretreated with	Amount of T-2 (mg/kg)	Survival Ratio[a]	LD_{50} (mg/kg)	Survival Time (hr)[b]
Saline	20	9/10		
	18	4/5	14.3[c]	21.3 (± 1.5)
	15	6/10	(11.4 - 16.2)	
	13	3/5		
	10	0/5		
MD_{11}	20	8/10		
2.0 mg	18	2/5	17.3	23.3 (± 1.0)
per mouse	15	3/10	(15.3 - 20.2)	
	13	1/5		
	10	0/5		
Control	20	10/10		
Antibody	18	5/5	13.7	
2.0 mg	15	4/10	(13.7 - 16.8)	22.2 (± 1.1)
per mouse	13	1/5		
	10	0.5		

[a]Number of dead mice/total number of mice.
[b]Average time between T-2 administration and death ± S.E.M.
[c]LD_{50} determined by point analysis. The numbers in parentheses represent the 95% confidence limits.

Table 3

INHIBITION OF HD_{11}-b BINDING TO T-2 BSA COATED WELLS BY T-2 METABOLITES

ug/ml	Metabolites No.									
	1	2	3	4	5	6	7	8	9	10
100	92[a]	61	0	0	0	71	36	0	98	0
50	89	38	0	0	0	66	25	0	99	0
25	87	31	0	0	0	65	29	0	98	0
12.5	83	22	0	0	0	58	15	0	98	0
6.2	76	0	0	0	0	37	18	0	99	0
3.1	52	0	0	0	0	55	21	0	98	0
1.6	14	0	0	0	0	36	8	0	99	0
0.8	20	0	0	0	0	35	21	0	99	0

[a]Mean percent inhibition of binding. The optical density of HD_{11}-b binding in the absence of inhibitor was 1.10. Free T-2 completely inhibited this binding at the lowest concentration of inhibitors tested (0.8ug/ml).

Table 4

INHIBITION OF DE8-B BINDING TO HD11 COATED WELLS BY T-2 METABOLITES

	Metabolite NO.										
(UG/ML)	1	2	3	4	5	6	7	8	9	10	11
50	84[a]	60	15	2	6	53	24	9	95	63	17
25	79	50	12	1	1	46	20	0	94	80	6
12.5	76	45	7	1	0	33	17	0	91	13	6
6.2	64	30	1	0	1	20	7	0	90	2	8
3.1	58	19	0	0	1	9	2	0	89	0	12
1.6	43	12	2	1	3	4	0	0	87	0	8
0.8	26	7	1	0	3	3	3	0	87	3	16
0.4	17	5	0	0	2	1	0	0	85	0	17

a Percent inhibition of binding. The optical density of DE8-b binding in the absence of inhibitor was .944.
Free T-2 completely inhibited this binding at a concentration of .05ug/ml.

Table 5

Administration of DE8 Anti-Idiotype Protects Mice Against T-2 Toxicity In-Vivo

Mice immunized with	T-2 Mycotoxin (ug/ml)	Survival ratio[a]
Control Anti-Id	5.0	3/5
	10.0	4/5
	15.0	5/5
	20.0	5/5
DE8 Anti-Id	5.0	0/4
	10.0	1/5
	15.0	0/5
	20.0	0/5

[a]Number of dead mice/total number of mice tested. T-2 mycotoxin was administered intradermally.

Table 6

Specificity of monoclonal anti-TDT antibody by TDT-BSA ELISA

Anti-TDT mAb	Inhibitors (µg/ml)			
	PBS	STX	TDT	
		100	100	0.05
TD2C5	0.289	0.303(0)	0.029(90)	0.150(48)
TD13a1	0.336	0.307(8)	0.011(92)	0.251(25)

Antibody was incubated for 1 hr at 37° in the presence of PBS, STX, or TDT at various concentrations prior to addition to an ELISA plate coated with 10 µg/ml of TDT-BSA as the solid phase. Selected inhibitor concentrations are displayed for ease of comparison. Data are the mean of duplicate OD_{410nm} determinations and the numbers in parentheses represent the percent inhibition of binding.

TABLE 7

Comparison of baseline and experimental nerve response amplitudes (in millivolts) in the presence or absence of TDT and/or TD2C5 anti-TDT antibody

Treatment (n = 6)	Baseline	5 min	10 min
Control	1.29 ± .23	1.20 ± .25	1.10 ± .18
TDT	1.31 ± .23	0.49 ± .12*	0.29 ± .07*
EXP 1	1.07 ± .08	0.96 ± .13	0.85 ± .08
EXP 2	1.27 ± .10	1.06 ± .13	0.79 ± .20
EXP 3	1.26 ± .08	0.69 ± .13*	0.40 ± .09*

*Significantly different from baseline or control response, $p < 0.05$ (Newman-Kuels analysis).

Experimental treatment consisted of soaking exposed rat tibial nerve for 5 min in 5.55 μM TD2C5 alone (control), 10 μM TDT alone (TDT), 10 μM TDT plus 5.55 μM TD2C5 (EXP 1), 10 μM TDT plus 2.78 μM TD2C5 (EXP 2) or 10 μM TDT plus 0.55 μM TD2C5 (EXP 3). Baseline recordings were recorded every 30 sec for a 3 min period prior to addition of any compound. Antibody and toxin were mixed together 1 hr prior to adding to the nerve. Experimental measurements were performed at 5 and 10 min post addition of TDT and/or TD2C5.

TABLE 8

Specificity of Antibody Produced by Anti-STX Cell Lines by STX-BSA ELISA

	Competitors (µg/ml)			
Anti-TDT	PBS	TDT	STX	
mAb		100	100	0.5
S1A5	0.714	0.660(8)	0.056(92)	0.264(63)
S3E.2	0.338	0.414(0)	0.004(99)	0.206(39)

Antibody was incubated for 1 hr at 37°C in the presence of PBS, STX, or TDT at various concentrations prior to addition to an ELISA plate coated with 10 µg/ml of STX-BSA as the solid phase. Selected inhibitor concentrations are displayed for ease of comparison. Data are the mean of duplicate OD_{410nm} determinations and the numbers in parentheses represent the percent inhibition of binding.

Table 9

Comparison of Baseline and Experimental Nerve Response Amplitudes (in Millivolts) in the Presence and Absence of STX and/or Antibody

Treatment	Baseline	5 min	10 min
S1A5 control, n = 4	1.29 ± 0.23	1.03 ± 0.33	0.98 ± 0.44
STX, n = 4	1.23 ± 0.06	0.34 ± 0.11[a]	0.19 ± 0.08[a]
EXP 1, n = 3	1.52 ± 0.12	0.46 ± 0.09[a]	0.08 ± 0.06[a]
EXP 2, n = 3	1.14 ± 0.09	0.37 ± 0.11[a]	0.18 ± 0.07[a]
S3E.2 control, n =4	1.51 ± 0.12	1.49 ± 0.14	1.43 ± 0.13
STX, n = 4	1.43 ± 0.14	0.54 ± 0.15[a]	0.13 ± 0.06[a]
EXP 1, n = 4	1.49 ± 0.26	0.91 ± 0.31[a]	0.55 ± 0.11[a]
EXP 2, n = 5	1.46 ± 0.23	1.05 ± 0.24[a]	0.54 ± 0.13[a]
EXP 3, n = 3	1.37 ± 0.17	1.21 ± 0.15	0.59 ± 0.20[a]

[a]Significantly different from baseline or control response, $p < 0.05$, Neuman-Kuels.

Experimental treatment consisted of soaking exposed rat tibial nerve for 5 min in 10 μM of antibody alone (control), 1 μM STX alone (STX), 1 μM STX plus 5 μM antibody (EXP 1), 1 μM STX plus 10 μM antibody (EXP 2), or 1 μM plus 30 μM antibody (EXP 3). Baseline recordings were made every 30 sec for a 3 min period prior to additon of any compound. Antibody and toxin were mixed for 1 hr together prior to adding to the nerve. Experimental measurements were performed at 5 and 10 min post addition to toxin or antibody.

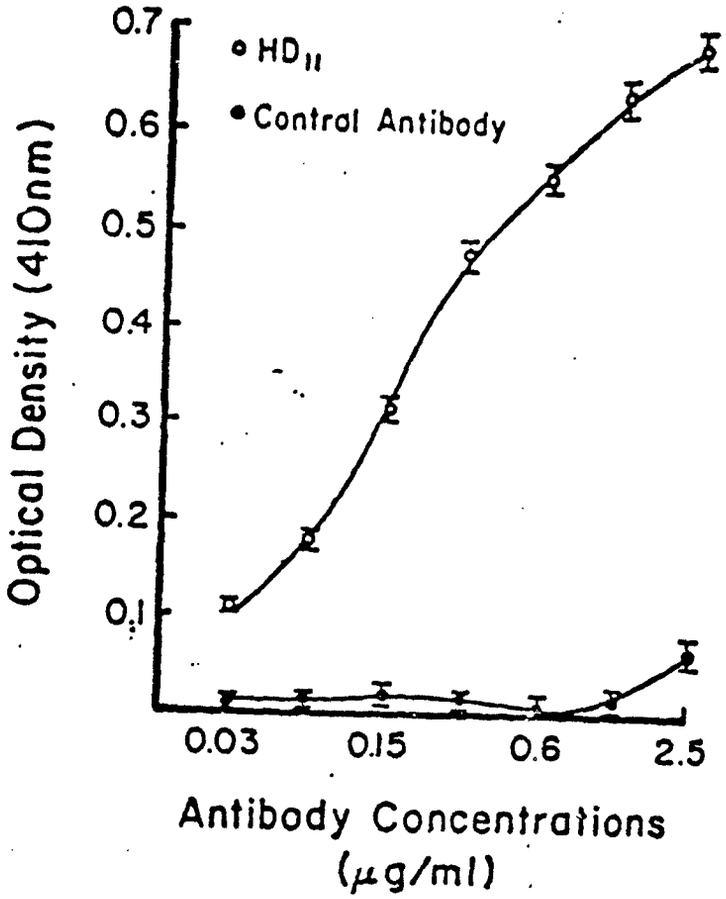

0.7
0.6
0.5
0.4
0.3
0.2
0.1
Optical Density (410nm)
HD11
Control Antibody
0.03
0.15
0.6
2.5
Antibody Concentrations
(μg/ml)

Figure 1

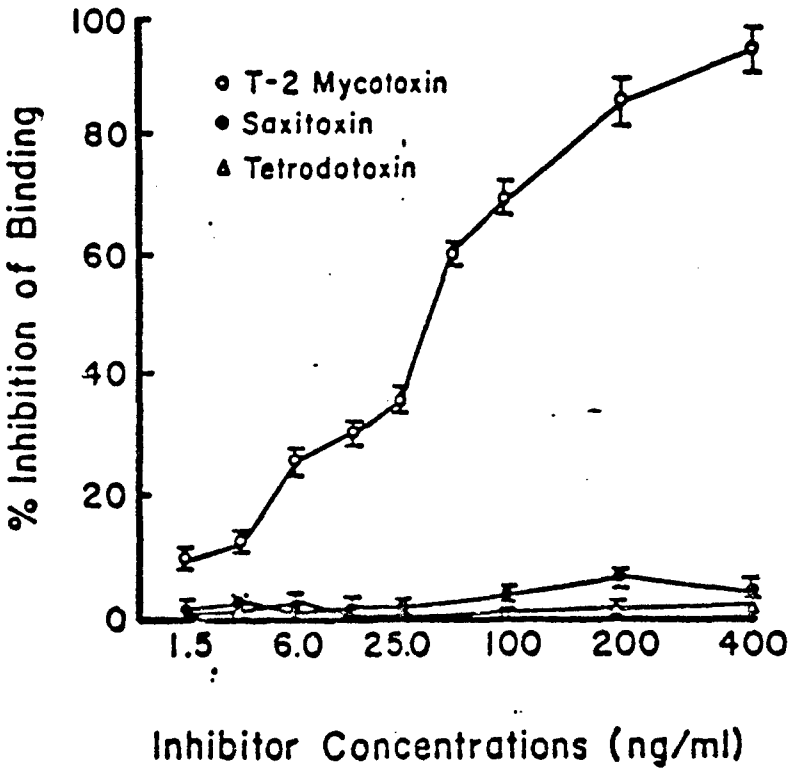

100
80
60
40
20
0
% Inhibition of Binding
T-2 Mycotoxin
Saxitoxin
Tetrodotoxin
1.5
6.0
25.0
100
200
400
Inhibitor Concentrations (ng/ml)

Figure 2

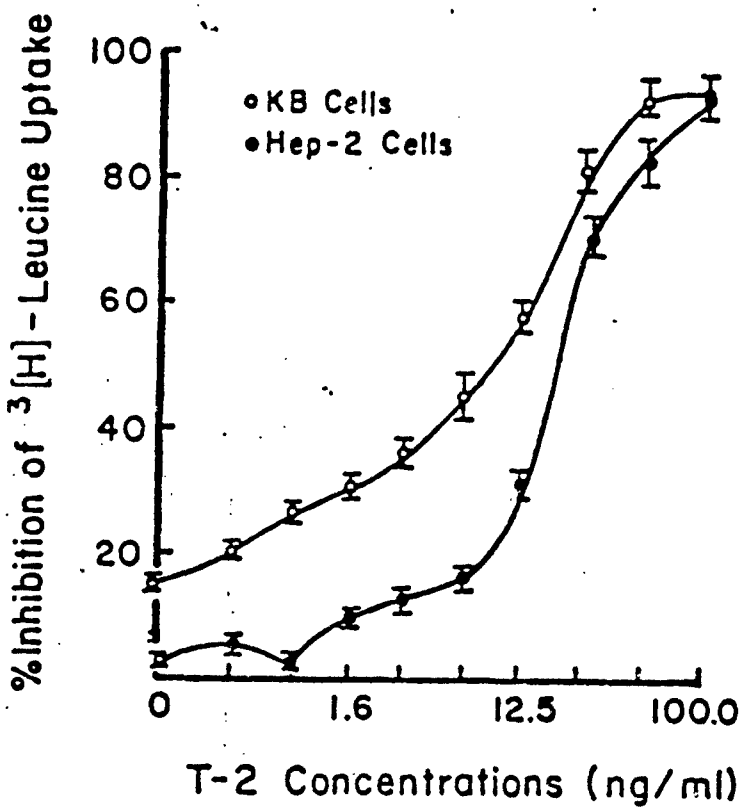

%Inhibition of ^{3}[H]-Leucine Uptake
100
80
60
40
20
0
1.6
12.5
100.0
KB Cells
Hep-2 Cells
T-2 Concentrations (ng/ml)

Figure 3

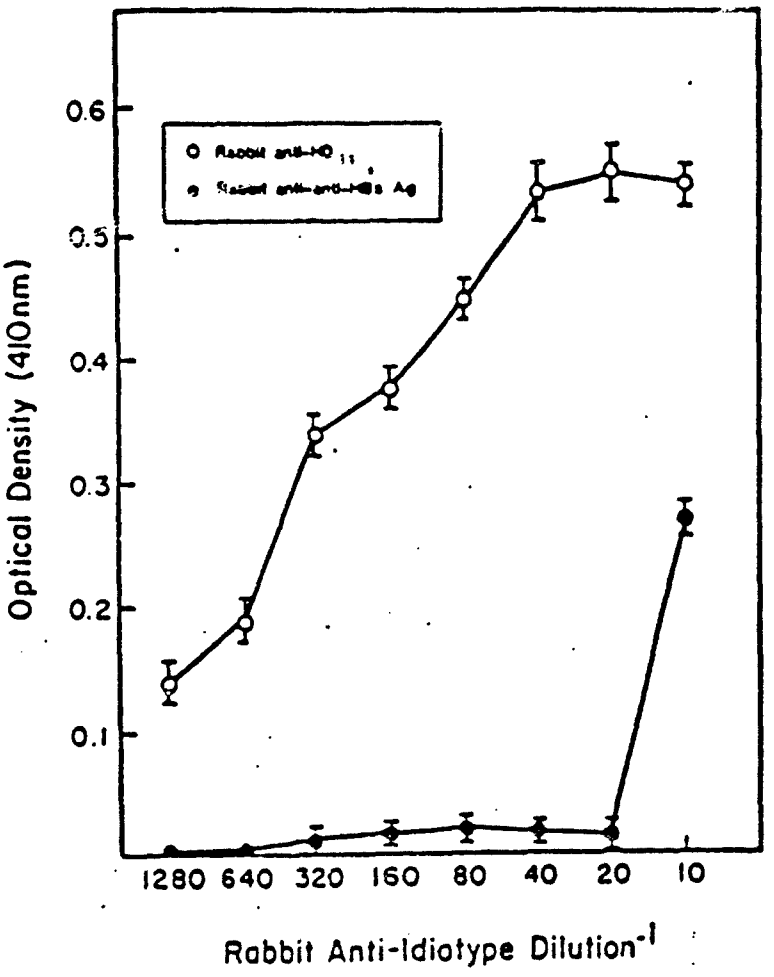

0.6
0.5
0.4
0.3
0.2
0.1
Optical Density (410nm)
1280 640 320 160 80 40 20 10
Rabbit Anti-Idiotype Dilution-1

Figure 4

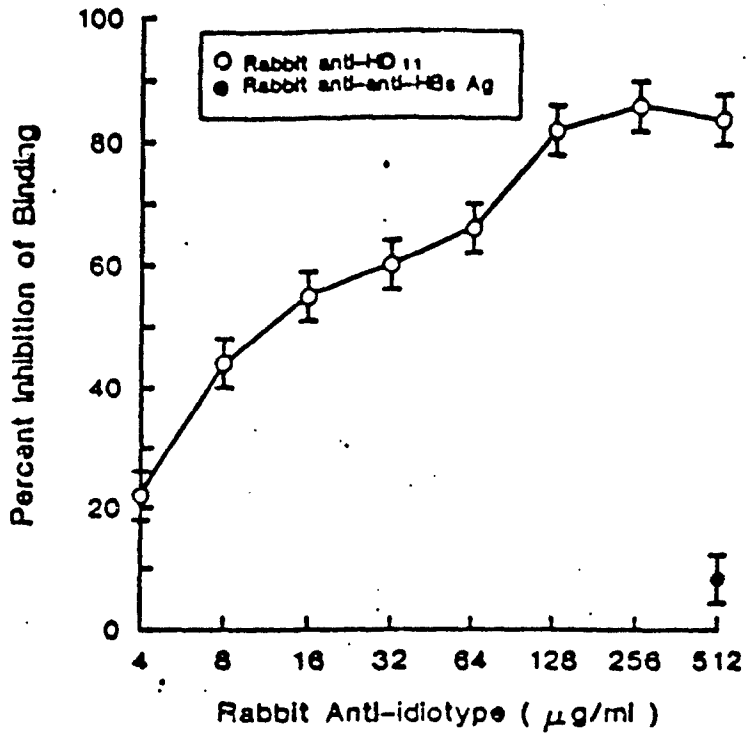
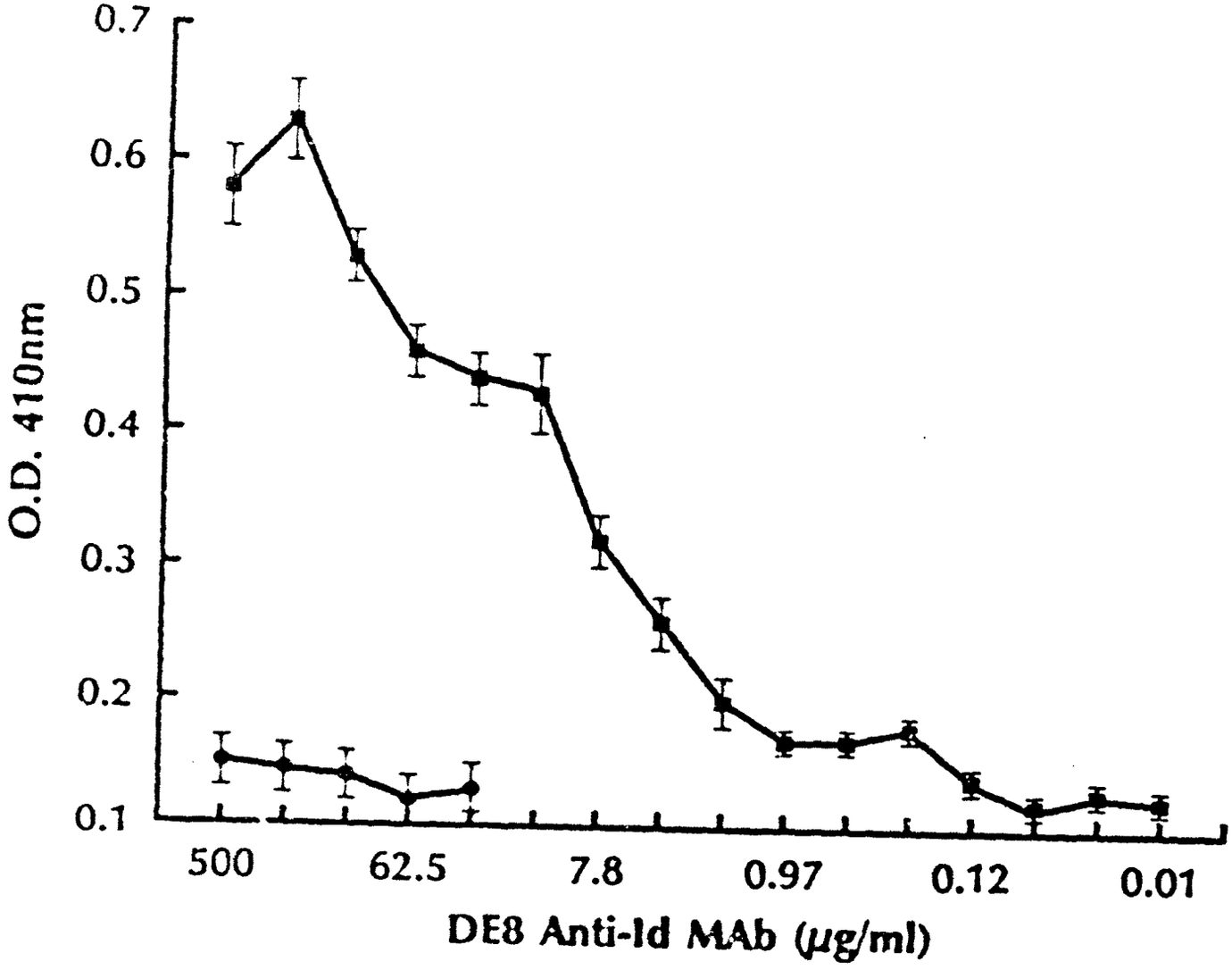

100
80
60
40
20
0
Percent Inhibition of Binding
O Rabbit anti-HD 11
● Rabbit anti-anti-HBs Ag
4
8
16
32
64
128
256
512
Rabbit Anti-idiotype (μg/ml)

Figure 5


0.7
0.6
0.5
0.4
0.3
0.2
0.1
O.D. 410nm
500
62.5
7.8
0.97
0.12
0.01
DE8 Anti-Id MAb (μg/ml)

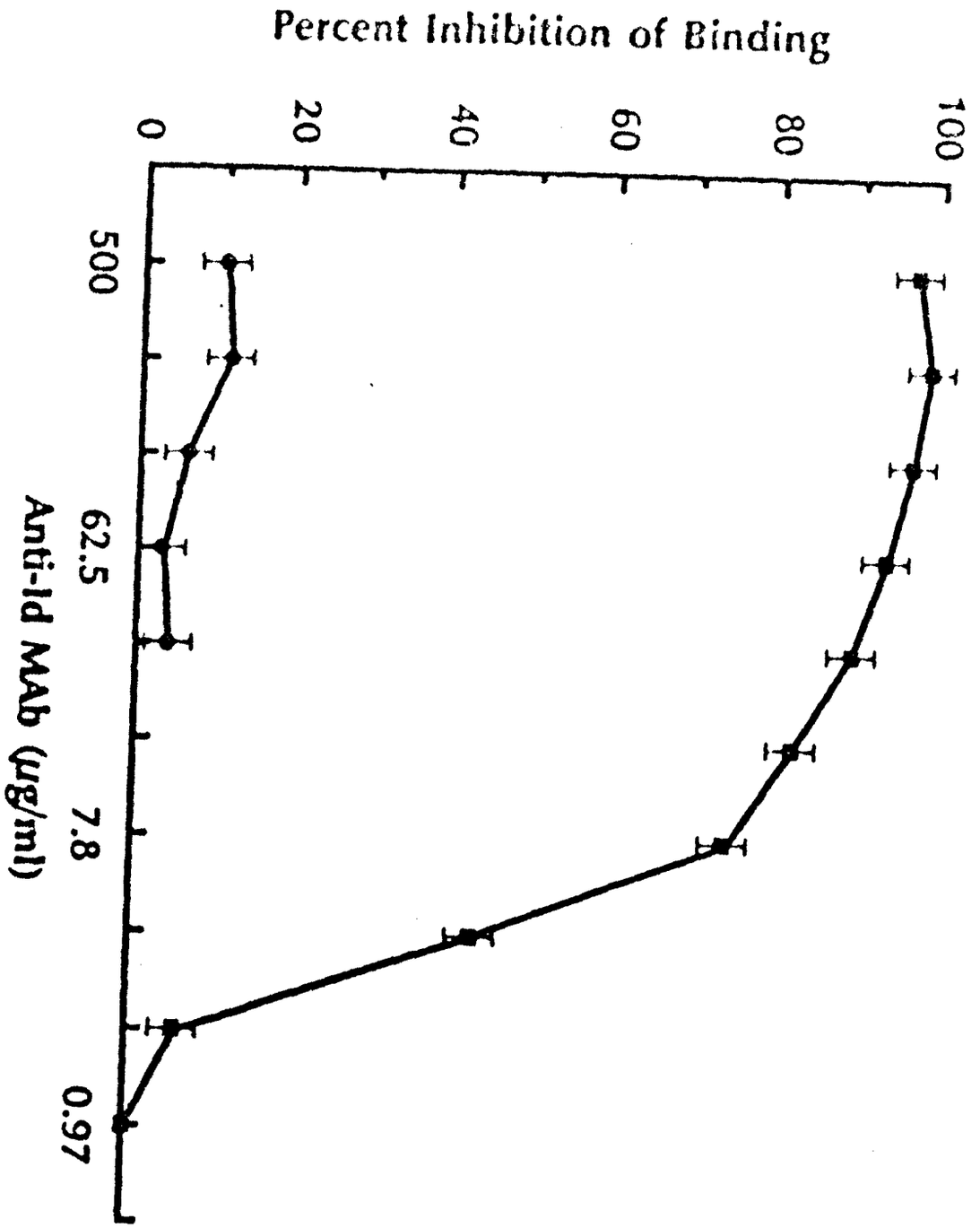

Percent Inhibition of Binding
0
20
40
60
80
100
500
62.5
7.8
0.97
Anti-Id MAb (µg/ml)

Figure 7

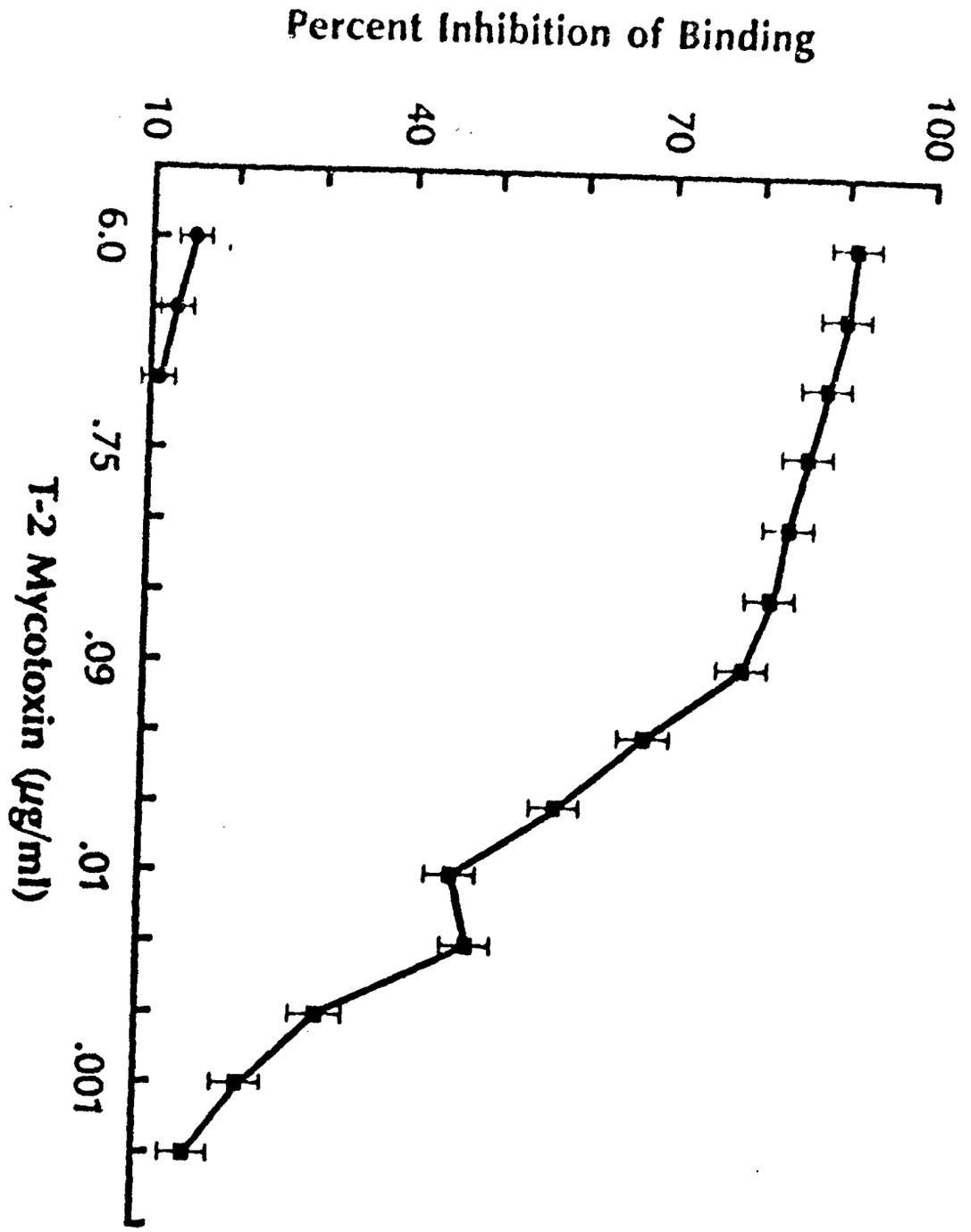

Percent Inhibition of Binding
10
40
70
100
6.0
.75
.09
.01
.001
T-2 Mycotoxin (µg/ml)

Figure 8

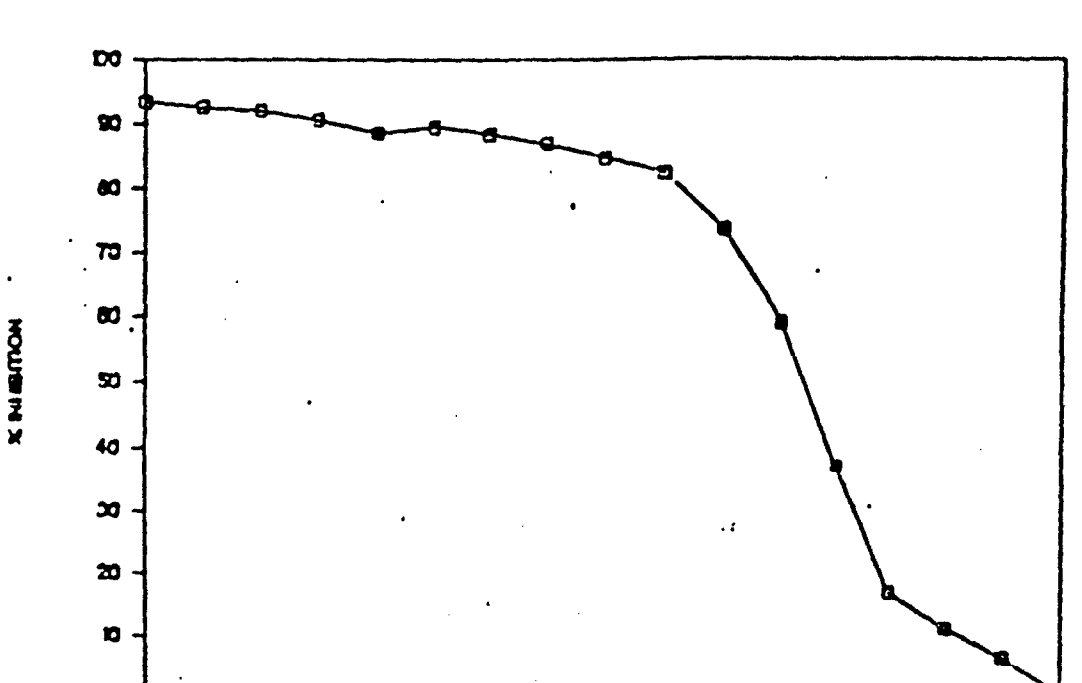

% INHIBITION
100
90
80
70
60
50
40
30
20
10
0
200
25
3.1
0.78
0.04
0.005
T-2 CONCENTRATION (UG/ML)

Figure 9

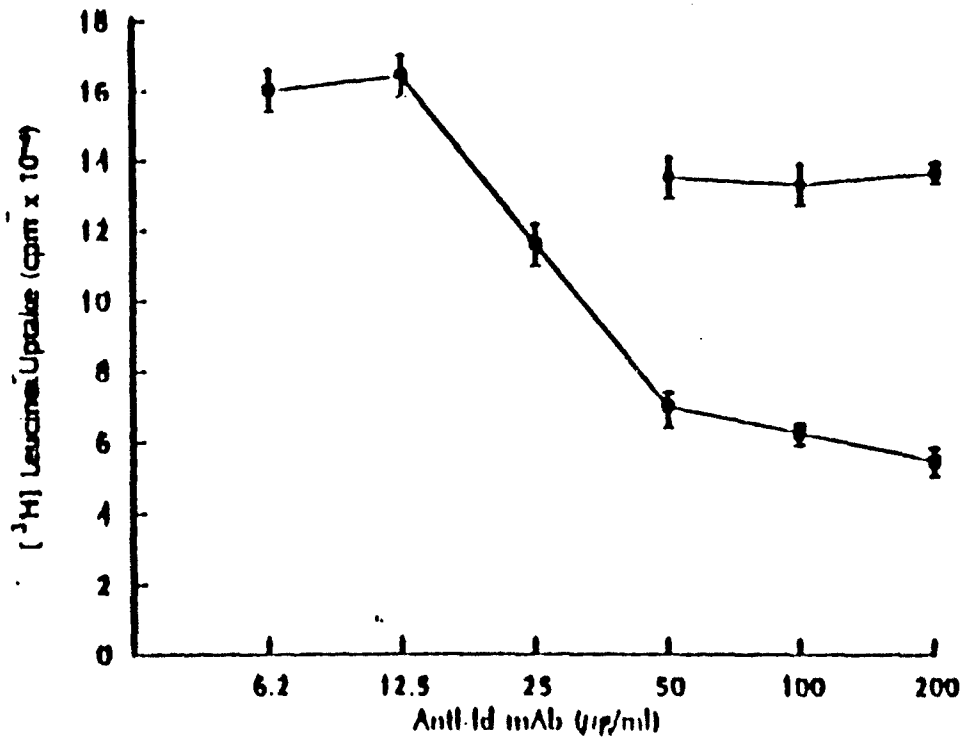


Figure 10

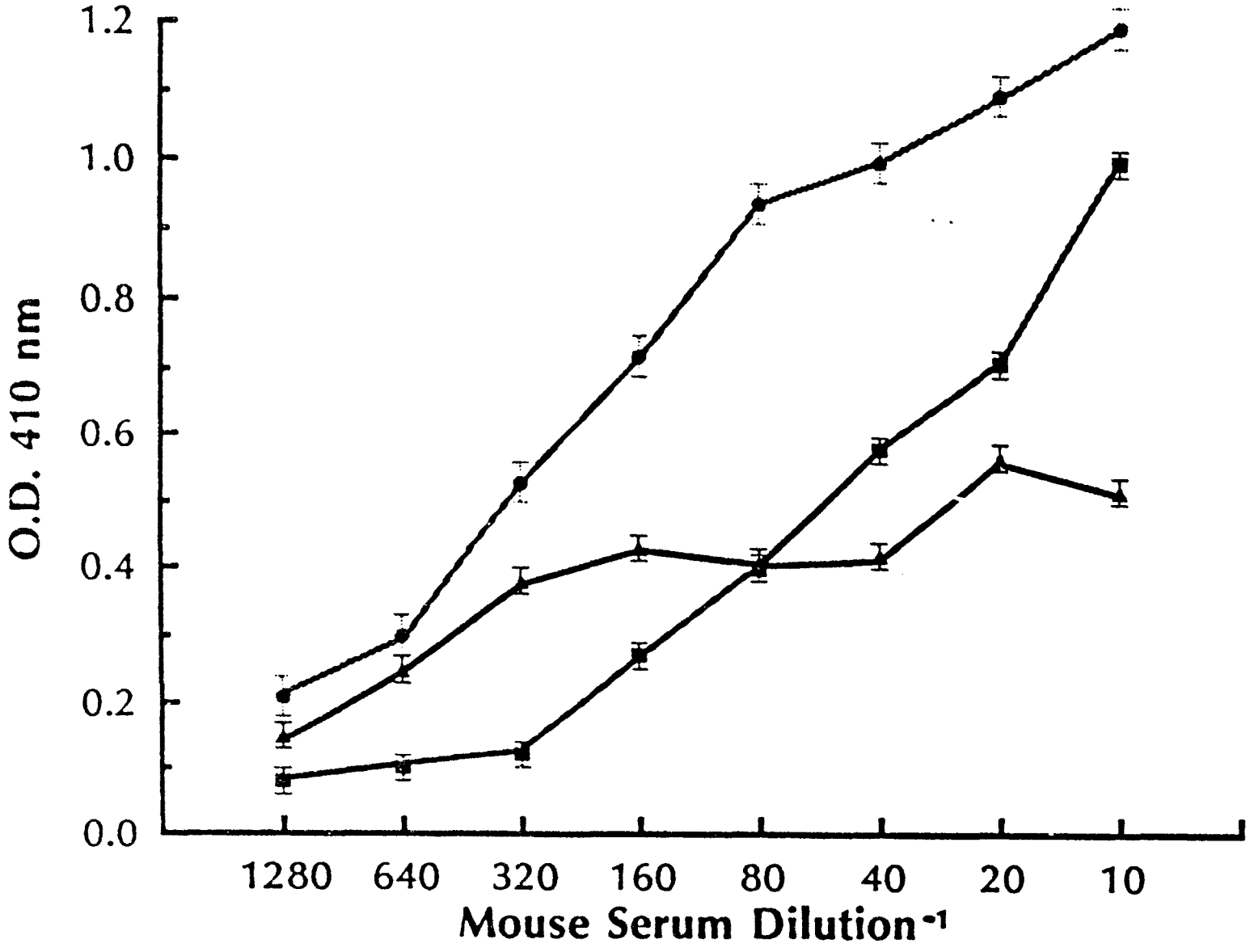

1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D. 410 nm
1280
640
320
160
80
40
20
10
Mouse Serum Dilution⁻¹

Figure 11

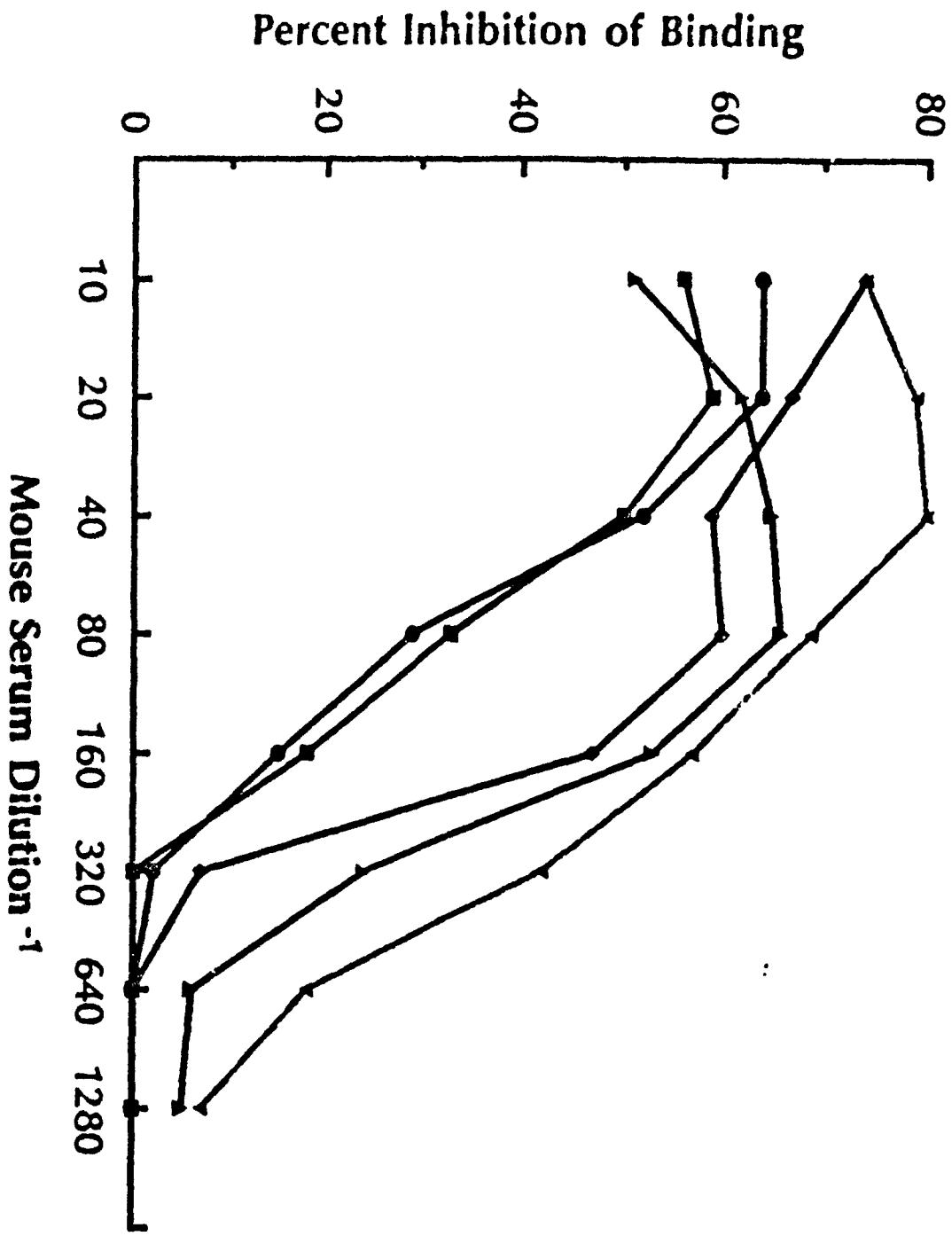

Percent Inhibition of Binding
0
20
40
60
80
Mouse Serum Dilution -1
10
20
40
80
160
320
640
1280

Figure 12

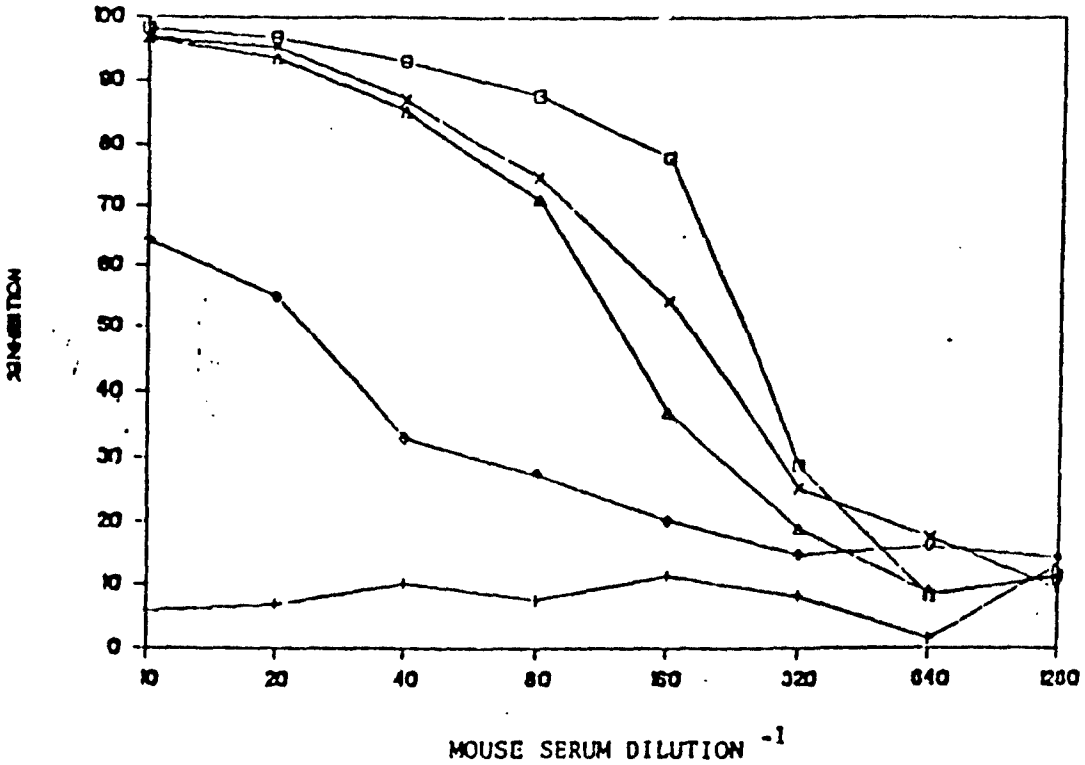

100
90
80
70
60
50
40
30
20
10
0
10
20
40
80
160
320
640
1280
MOUSE SERUM DILUTION -1

Figure 13

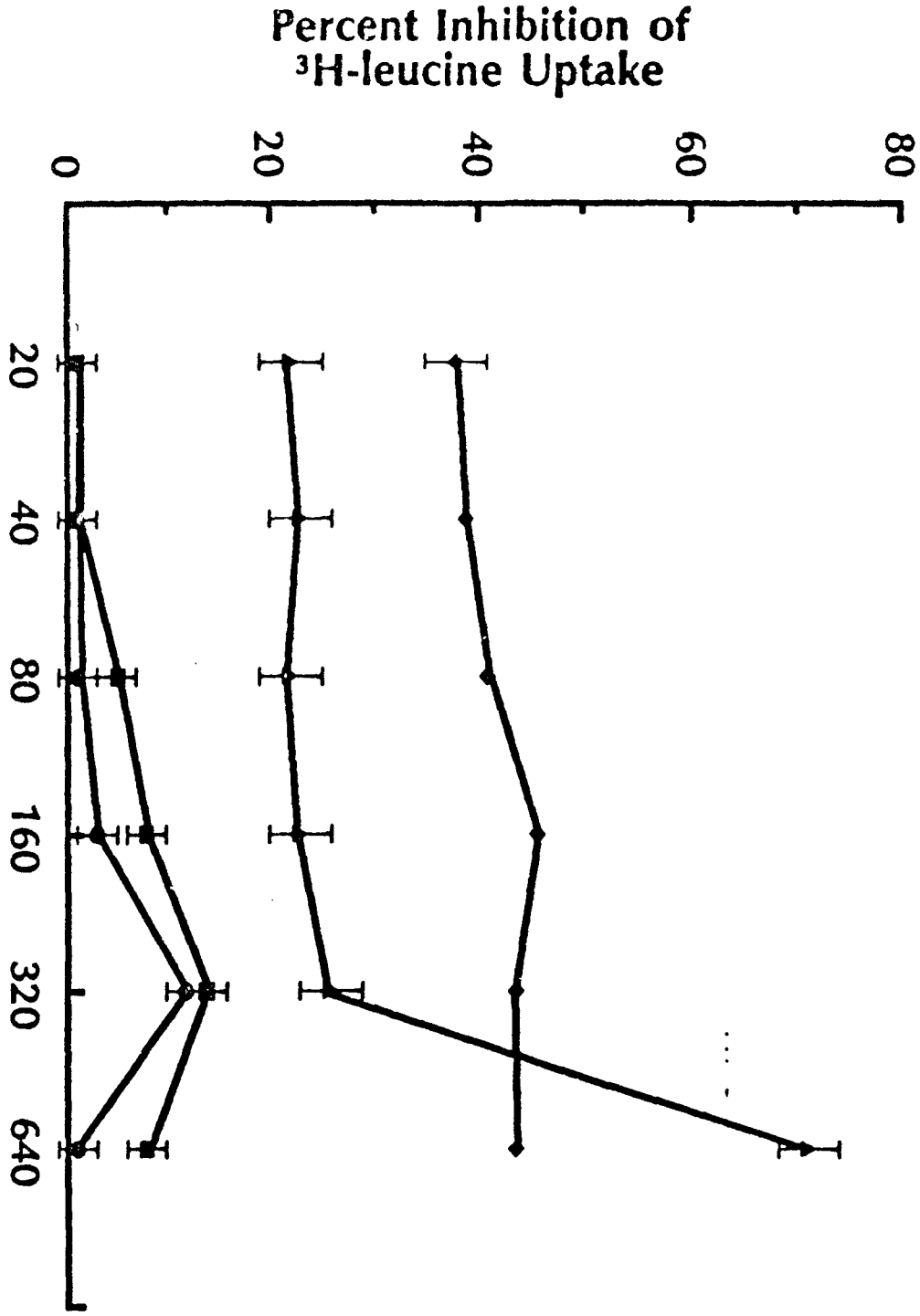

Percent Inhibition of
³H-leucine Uptake
0
20
40
60
80
Mouse Serum Dilution⁻¹
20
40
80
160
320
640

Figure 14

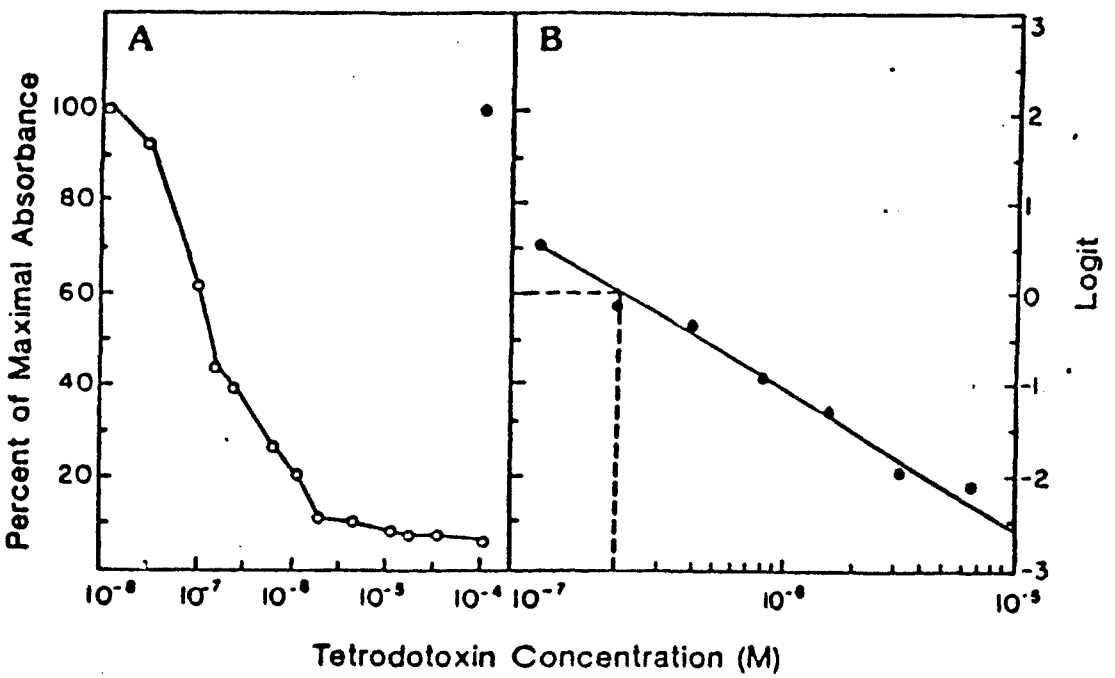

A
B
Percent of Maximal Absorbance
100
80
60
40
20
3
2
1
0
-1
-2
-3
Logit
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-7}
10^{-6}
10^{-5}
Tetrodotoxin Concentration (M)

FIGURE 15

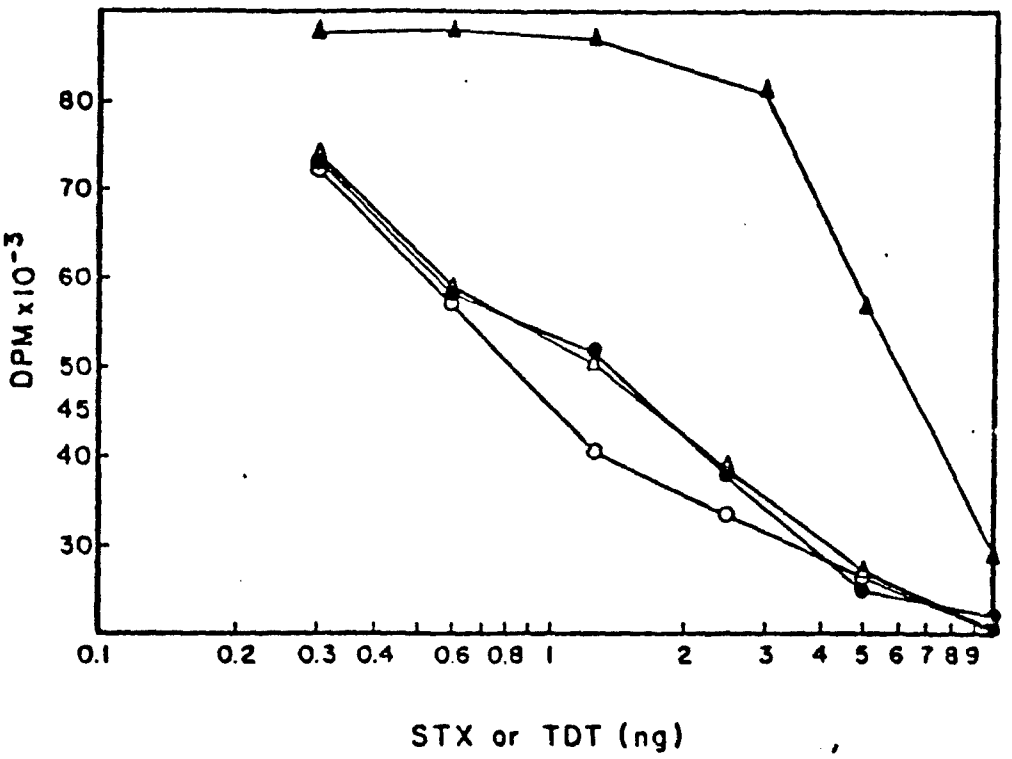

DPM x10⁻³
80
70
60
50
45
40
30
0.1
0.2
0.3
0.4
0.6
0.8
1
2
3
4
5
6
7
8
9
STX or TDT (ng)

FIGURE 16

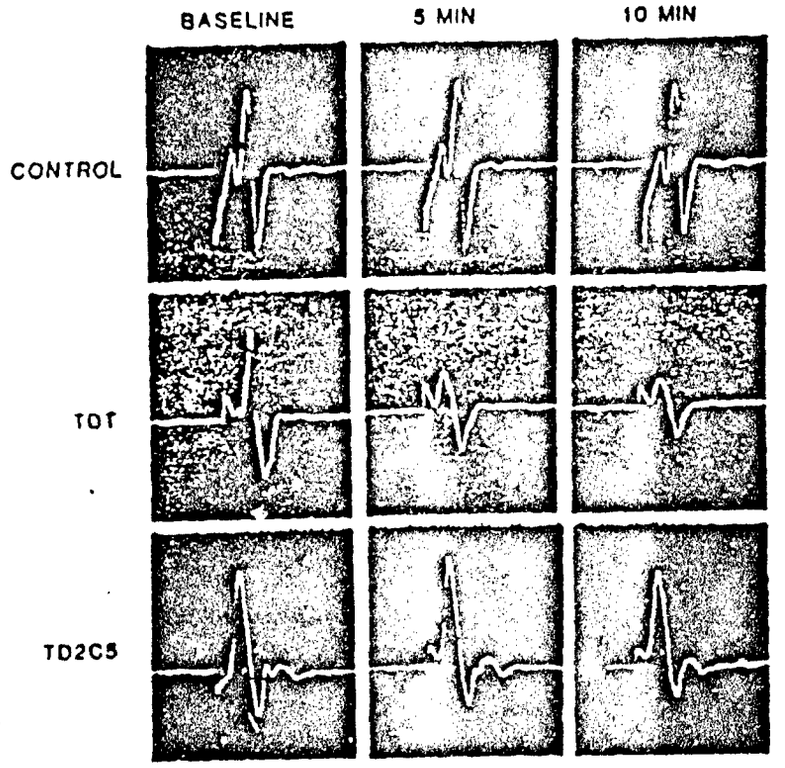


FIGURE 17

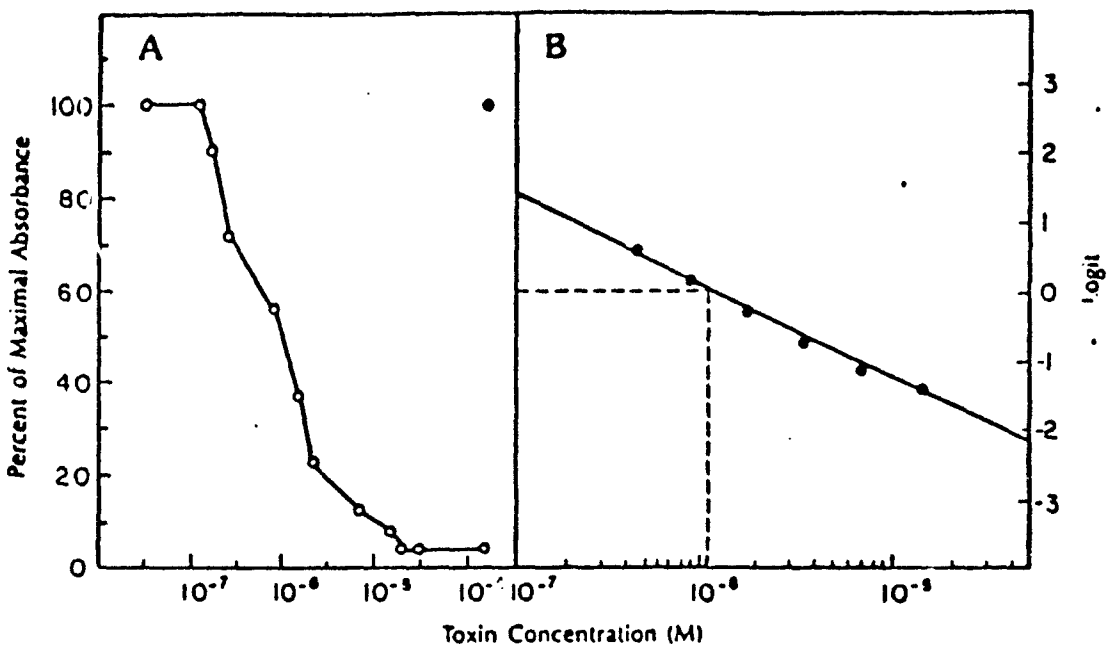

A
B
Percent of Maximal Absorbance
100
80
60
40
20
0
3
2
1
0
-1
-2
-3
Logit
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-7}
10^{-6}
10^{-5}
Toxin Concentration (M)

FIGURE 18

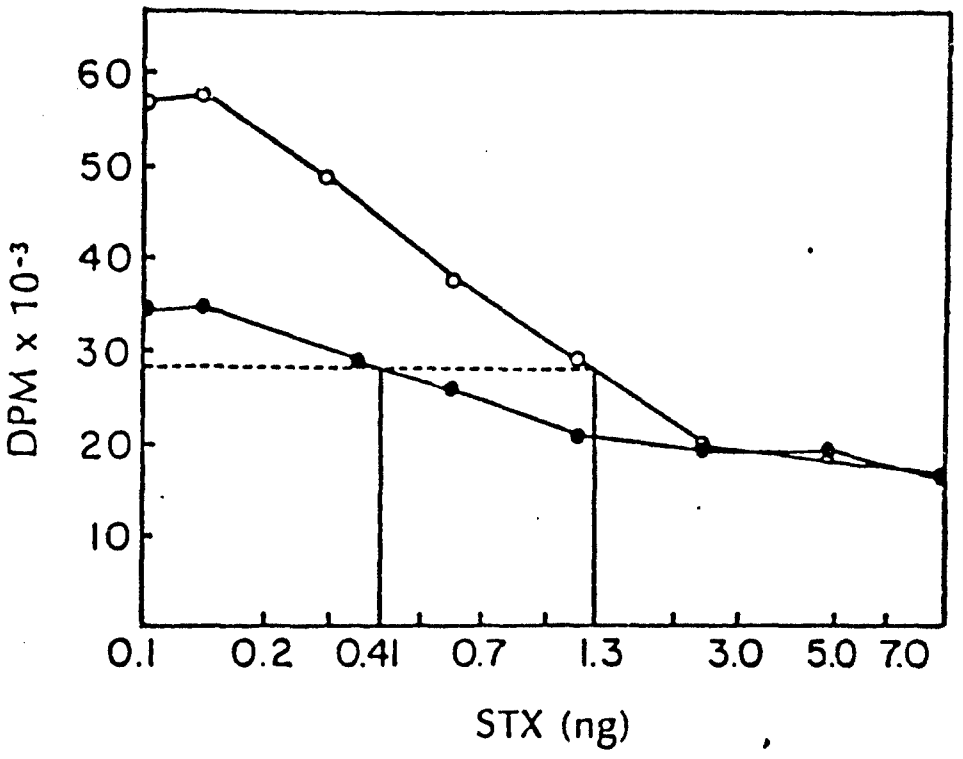


Figure 19

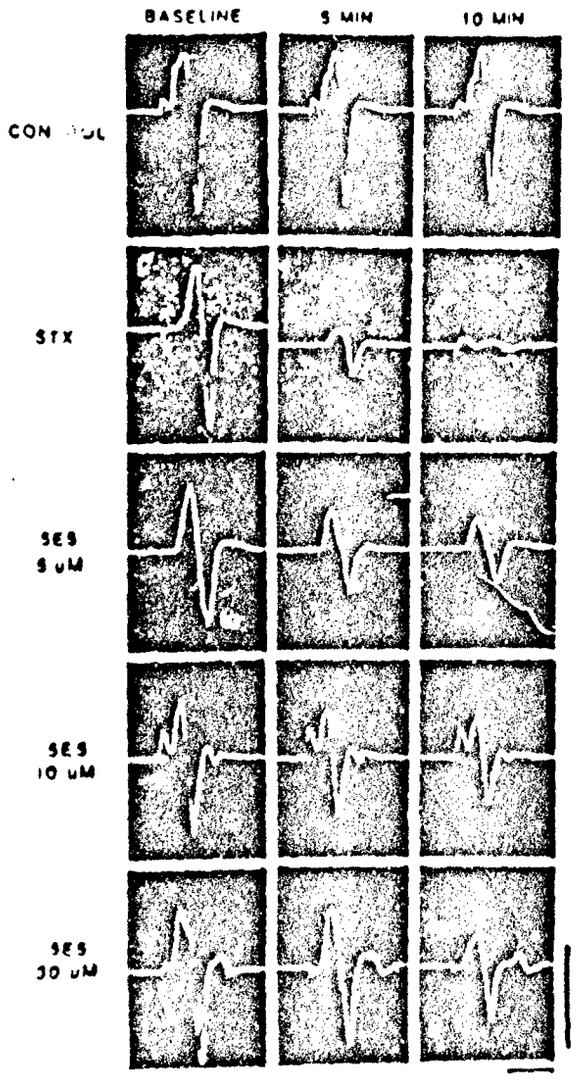


FIGURE 20

APPENDIX I

ETIOLOGIC AGENTS

1. HT-2 Toxin
2. 3' OH T-2 Toxin
3. 3' OH HT-2 Toxin
4. Neosalaniol Toxin
5. Tetracl Toxin
6. Diacetylscirpenol Toxin
7. Deoxyhiavalenol Toxin
8. Deoxyverrucarol Toxin
9. Acetyl T-2 Toxin
10. Verrucarol Toxin
11. Labelled "Triol"